Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Scienze e Tecnologie Agrarie, Ambientali e Alimentari

Ciclo XXVIII

Settore Concorsuale di afferenza: 07/D1

Settore Scientifico disciplinare: AGR/12

Sensitivity studies of *Plasmopara viticola* to Carboxylic Acid Amide fungicides: *in vivo* test and molecular studies of Pv*CesA3* gene

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Esame finale anno 2016

Alma Mater Studiorum – Università of Bologna

Research Doctorate in

Agricultural, Environmental and Food Science and Technology

XXVIII Cycle

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Final exam year 2016

"..Und alles zusammen, alle Stimmen, alle Ziele, alles Sehnen, alle Leiden, alle Lust, alles Gute und Böse, alles zusammen war die Welt. Alles zusammen war der Fluß des Geschehens, war die Musik des Lebens."

Herman Hesse – Siddharta

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Contributions:

Collina M, Nanni IM, Contaldo N, Brunelli A. Sensitivity to Carboxylic acid amide fungicides of *Plasmopara viticola* populations in Northern Italy. Modern Fungicides and Antifungal Compounds VII, 2013 (p.273)

Nanni IM, Pirondi A, Ciriani A, Brunelli A, Collina M. Molecular and Biological methods to evaluate the sensitivity to Carboxylic Acide Amide (CAA) fungicides of *Plasmopara viticola* populations.7th GDPM, 2014.Abstract book (p.160)

Nanni IM, Pirondi A, Mancini D, Brunelli A, Collina M. New findings about the sensitivity of *Plasmopara viticola* to CAA fungicides. XVIII International Plant Protection Congress, 2015**.** Abstract book (p.623)

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Publications:

Nanni IM, Pirondi A, Contaldo N, Collina M. Screening of sensitivity to mandipropamid of *Plasmopara viticola* populations from Italian vineyards utilizing molecular and biological methods (submitted to Letters in Applied Microbiology)

Nanni IM, Pirondi A, Mancini D, Stammler G, Gold R, Ferri I, Brunelll A,Collina M. (2015) Differences in the efficacy of carboxylic acid amide fungicides against less sensitive strains of *Plasmopara viticola. Pest Management Science* DOI10.1002/ps.4182

ABSTRACT

Many oomycetes species are pathogens of plants, animals and humans; some of them are well studied because they cause significant economic losses in agriculture and acquaculture. The cell wall of oomycetes consists mainly of cellulose, β- (1,3) and β- (1,6)- glucan and in some species a small amount of chitin is also present. The biosynthesis of cellulose in oomycetes is still poorly studied and therefore misunderstood, although it's the target of some fungicides that inhibit this process, such as carboxylic acid amides. Since 2010, single amino acid exchanges in *CesA3* protein conferring CAA resistance in *Plasmopara viticola* have been identified. In this work, we initially provide the first evidence of the presence of mandipropamid resistant populations of *Plasmopara viticola* in commercial vineyards in Italy (paper I). We continue by studying the different activity of four CAAs fungicides (benthiavalicarb, dimethomorph, iprovalicarb, mandipropamid) toward *P. viticola* resistant strains (paper II). The results show that the G1105S mutation affects all four CAAs, but its impact is varied. These results confirm that they are cross resistant, although many gaps in the mode of action are still present. In order to confirm our previous findings, we performed a microscopical base method to assess the sensitivity of four CAAs, and preliminary microscopical data confirmed the different activity toward the CAA-resistant and CAA-sensitive populations (chapter ten). Furthermore, we present a study (chapter eleven) in which we randomly selected samples from paper I, in order to test them using a different approach, simulating a scenario more close to the field. With this test, we were able to confirm the data presented in the paper I.

Key words: Oomycete, *Plasmopara viticola* ; Fungicide Resistance; *CesA* genes; Pv*CesA3* gene; Cellulose synthase

1 OUTLINE OF THE THESIS

The aim of this work was to analyze the sensitivity of *Plasmopara viticola* to CAA fungicides and to detect the G1105S mutation located on the Pv*CesA3* gene, which confers resistance to CAA fungicides. The techniques applied to develop this purpose were both biological and molecular. To guide the reader through the work presented here, the introduction starts by describing the phylogeny and the biology of the oomycetes, then continues with a focus on the pathogenic oomycetes and in particular on *Plasmopara viticola.* An explanation about the principal content of the oomycetes cell wall, such as cellulose, is given. Since cellulose is essential for the survival of the oomycetes, the biosynthesis of the cellulose is the main target for the CAA fungicides; therefore, in the following chapters, cellulose and its biosynthesis are presented in detail. During this research, I had the opportunity to note the different behaviors that were occurring among the CAA fungicides studied, in particular between dimethomorph (cinnamic acid amide) and mandipropamid (mandelic acid amide) toward *P.viticola.* This interesting "finding" was the inspiration for other work that is still ongoing, aimed at better understanding the affects of these differences on activity in this class of fungicides. In chapter eight, I will present a molecular method (RFLP – PCR) that was developed in order to quickly detect the single point mutation G1105S occurring on Pv*CesA3* gene. The sensitivity to CAA fungicides was also studied using the traditional leaf-disc bioassay method. In chapter nine, I will present a focused study to underline the differences in the response of CAA *P.viticola* resistant trains toward different CAAs (benthiavalicarb, dimethomorph, iprovalicarb, mandipropamid). To further examine and confirm the results obtained in the previous experiments, we performed a fluorescent microscopical–based technique, testing CAA-sensitive and CAA-resistant populations at different CAA concentrations (chapter ten). Furthermore, seedling tests were carried out in order to confirm the mandipropamid activity and compare it with the disc leaf assay test proposed in chapter eight.

2 INTRODUCTION TO OOMYCETES

2. 1 Phylogeny and general features of Oomycetes

Oomycetes are grouped in the kingdom of Chromista / Stramenopiles or Straminipila according to the formal diagnosis of the kingdom by Dick in 2001 and they are commonly referred to as water moulds and downy mildews; in fact, among Stramenopiles, we found heterokont algae such as diatoms, brown and golden algae (Cavalier-Smith,1993, Walker and Van West, 2007). Genomic analyses like SSU and LSU rDNA have shown that oomycetes have their evolutionary origins in the sea, evolving from *Haptocarpa s*p, an obligate parasite of rotifers and nematodes, and from *Eurychasma dicksonii,* an obligate parasite of brown seaweeds, characterized by the production of swollen holocarpic talli (Beakes *et al*; 2012). In addition, other early diverging genera include holocarpic *Olpidiopsis* sp, which are parasites of red seaweeds (Sekimoto *et al*; 2008). As previously mentioned, the majority of these taxa are obligate parasites that cannot be cultured without their hosts, and this trait is peculiar to most oomycetes. The current taxonomic classification of oomycetes is due to the significant work of Sparrow in 1960 and 1976 and Dick in 2001. They proposed that all oomycetes could be assigned to two main taxonomic groups, one included the *Saprolegnian* water moulds order (*Eurychasmales, Leptomitales and Saprolegniales*) and the other one comprised of plant pathogenic "peronosporalean orders" (*Rhipidiales, Pythiales and Peronosporales*). (Fig 1) The peronosporalean branch, which also encompasses the *Rhipidiales* and *Albuginales* as well as Peronosporales, (Fig 2) represents the main divergence within the lineage (Beakes *et al*; 2012)

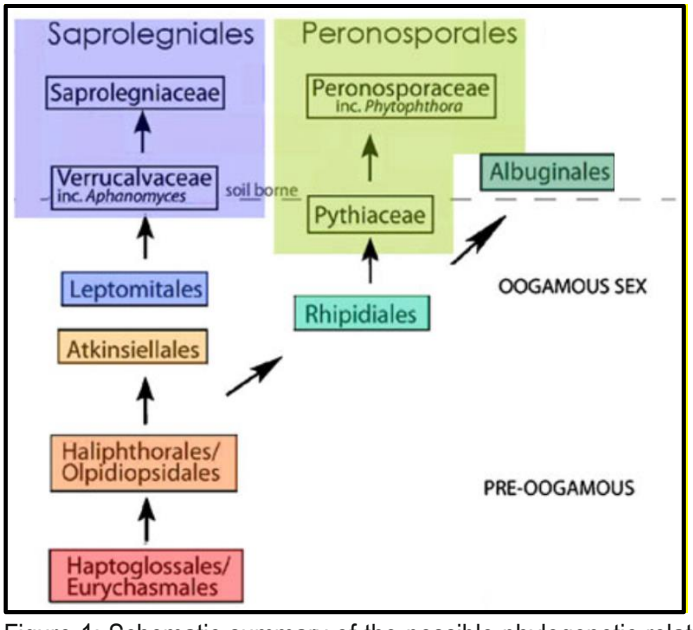

Figure 1: Schematic summary of the possible phylogenetic relationships between the main oomycete orders and families, based on current molecular data, modified from an original diagram by Beakes *et al*; 2012.

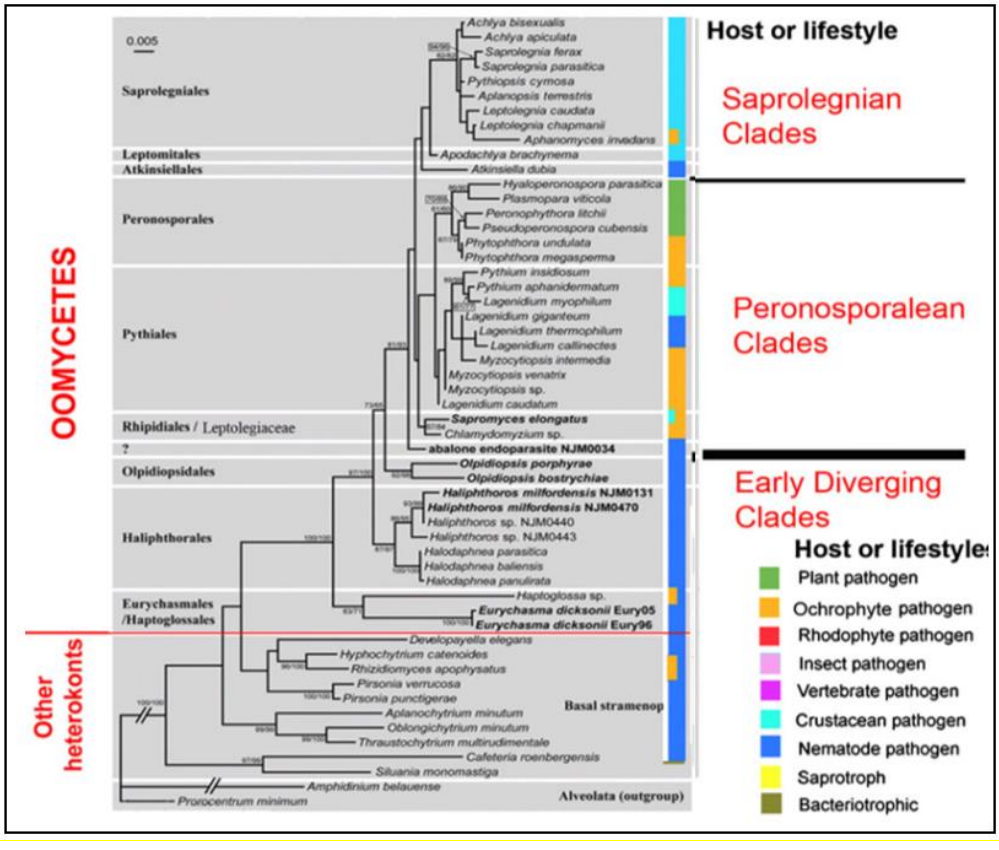

Figure 2: Figure modified from Sekimoto *et a*l; 2008 and Beakes *et al*; 2012. Maximum likelihood tree based on 54 SSU rRNA tree sequences of oomycetes, other stramenopiles with two members of the alveolata as an outgroup. The main oomycete order clades are labelled on right. The left hand bar map onto this tree the host or life style respectively.

According to Bakes *et al*. (2012), oomycetes have been placed in the phylum pseudofungi together with the *Hyphochytrids Pirsonia,* and *Developayella*, but this new classification is still under debate. The oomycetes have a number of remarkable biological features that distinguish them from other eukaryotic microorganisms. Oomycetes show filamentous growth in their vegetative stage, produce mycelia and form spores for asexual and sexual reproduction. Oomycetes are also characterized by having mitochondria with tubular cristae and they synthesize lysine via the diaminopimelic acid pathway (Vogel, 1965, Powell *et al*; 1985). The asexual spores can undergo cytoplasmic cleavage, resulting in the formation of zoospores (Latijnhouwers *et al*; 2003). Oomycete zoospores are single nucleated and wall-less cells that can swim with the help of flagella: one tinsel-anterior flagellum, and one whiplash-posterior flagellum (Walker and Van West, 2007). The cell wall is mainly composed of β- (1,3)- glucan polymers and cellulose, diverse from true fungi that contain chitin instead of cellulose. Many Oomycetes are partial sterol auxotrophs, their membrane contain lipid with unusual structures and long-chain fatty acids (Latijnhouwers *et a*l; 2003). Other singular characteristics include the energy storage carbohydrate mycolaminarin, a β- (1,3)- glucan that is also found in kelp and diatoms (Erwin *et al*; 1993). As their vegetative stage is diploid, and homologous recombination has not been known to occur, oomycetes are less adaptive to genetic manipulation than many fungi; for this reason only few genome sequences have been published so far, belonging to the genus *Phytophthora*, such as *Phytophthora infestans*, *P. ramorum* and *P. sojae* (Haas *et al*; 2009; Tyler *et al*; 2006). The genome size of oomycetes varies among the species, from 18 to 37Mb (Judelson, 2012) and recent molecular results have shown that the genome of oomycetes is characterized by an abundance of repetitive sequences (Mao and Tyler, 1996; Lamour *et al*; 2007). Meanwhile, oomycetes genome studies are still ongoing and they will provide further knowledge about this interesting class of organisms that are still understudied in every aspect.

2.2 Pathogenic Oomycetes

The multitude of saprophytic oomycetes inhabit primarily aquatic and moist soil habitats. They have a positive effect on nutrient cycling through their key role in the decomposition and recycling of organic matter (Kamoun, 2003; Mergulis and Schwartz, 2000). Plant – pathogenic oomycetes cause devastating diseases in numerous crops and native plants. They include more than sixty species of the genus *Phytophthora,* the most devastating pathogens of dicotyledonous plants. *Phytophthora* sp cause enormous economic damage to important crop species such as potatoes, tomatoes, peppers, soybeans, and alfalfa in addition to damaging natural ecosystems (Kamoun, 2003; Agrios, 2005). The most important pathogenic oomycete is *Phytophtora infestans* which cause late blight, a ravaging disease that affects potatoes and tomatoes (Kamoun, 2003; Birch and Whisson,2001), causing between $3-5 billion per year in losses in potato production worldwide (Judelson and Blanco, 2005). Other economically important *Phytophthora* diseases include root rot in soybeans, caused by *Phytophthora sojae,* black pod in cocoa, caused by *Phytophthora palmivora* and *Phytophthora megacarya,* dieback and related root rot diseases in crops and native plant communities, caused by *Phytophthora cinnamomi,* and sudden oak death, caused by *Phytophthora ramorum.* Important oomycete plant pathogens also occur outside of the genus *Phytophthora,* including obligate biotrophs *Plasmopara viticola* (the agent of downy mildew in grapevines) and Albugo, Bremia and Peronospora species, which cause white rust and downy mildew in several crops. Other important pathogens among oomycetes are more than hundred species of the genus *Pythium*, abundantly present in soil and water, that cause different plant diseases, mainly in root tissue. Among oomycetes we also found animal pathogenic species in the genus *Saprolegnia*, which can cause losses in aquacultures and fisheries. *Pythium insidiosum* is known to infect various mammals, including humans, horses and dogs (Mendoza *et al*; 1993; Mendoza *et al*; 1996). *P. insidiosum* colonizes cutaneous and subcutaneous tissues and can invade blood vessels and bones, causing fatal lesions (Ravishankar *et al*; 2001). One genus, *Aphanomyces*, includes both plant and animal pathogenic

species (Agrios, 1997). A facultative parasitic, oomycete *Lagenidium giganteum,* infects the larval stage of many mosquito species and spore formations (Kamoun, 2003).

Table 1: Description of the major genera of pathogenic oomycetes (modified from an original table by Kamoun, 2003)

Genus	Host(s)	Disease description
Albugo	Plants	Obligate pathogens of plant, i.e white rust
Aphanomyces	Animals and Plants	Moslty pathogens of aquatic animals but also in plant, i.e plague in crayfish and root rot disease in pear
Bremia	Plants	Obligate pathogens of plant, i.e lettuce downy mildew
Lagenidium	Animals	Pathogenic species of aquatic animals i.e mosquito larvae disease
Peronospora	Plants	Obligate pathogen of plants, i.e downy mildew of grapes
Phythophtora	Plants	Plant pathogen, that cause some of the most severe diseases in plants, i.e late blight in potato
Pythium	Animals, Plants and microbes	Mostly plant pathogens, mycoparisites and species that infects humans, i.e pathogeinic fungi that infect plants, skin lesions in mammals
Saprolegnia	Animals	Pathogenic species of aquatic animals, i.e Saprolegniosis in fish

3 *Plasmopara viticola*

3.1 Origin and biology

The oomycete that causes downy mildew on grapevines was first collected by Schweinintz in the northeastern US in 1834 and classified as the fungus *Botrytis cana*. In 1848, Berkley and Curtis re-classified it as the new species *Botrytis viticola*. De Bary, who had successfully studied the life cycle and classification of the potato late blight fungus *Phytophthora infestans,* described the asexual and the sexual stages of the grape pathogen and placed it in the genus *Peronospora,* as *Plasmopara viticola* (De Bary, 1863). Only twenty years later, in 1886, Schroeter noted the clear differences between several of the *Peronospora* fungi and separated this genus into *Peronospora* and *Plasmopora*. Berlese and deToni, using Schroeder's classification system, renamed this microorganism *Plasmopara viticola* in 1888. Downy mildew is endemic on wild *Vitis* species of North America and it was probably inadvertently introduced into Europe in about 1875 with American grape cuttings used to replant the French vineyards destroyed by *Phylloxera.* The European wine grape *Vitis vinifera,* which had evolved in the absence of the downy mildew pathogen, was extremely susceptible to it and the pathogen began to spread all over

Europe, affecting crops. Downy mildew is still most destructive in Europe and in the eastern half of the United States, where it can cause severe epidemics every year. The pathogen affects the leaves, fruit, and shoots of grapevines. The first symptoms of downy mildew are usually seen on the leaves as soon as 5 to 7 days after infection (Fig 3). Foliar symptoms appear as pale yellow irregular spots on the upper surface of the leaves and, after suitably warm and humid nights, a white downy fungal growth of the sporangiophores will appear on the underside of the leaves. (The disease gets its name "downy mildew" from the presence of this downy growth). Later, the infected leaf areas are killed and turn brown. The spots often enlarge, coalesce to form large dead areas on a leaf, and frequently result in premature defoliation. All young grapevine tissues are particularly susceptible to infection. Infected grapes are quickly covered with the fungal growth, and may become distorted or die. If infection takes place after the berries are half-grown, the oomycete grows mostly internally; the berries become leathery and somewhat wrinkled and develop a reddish marbled to brown coloration (Fig 4). In late shoots infections, the shoots are not killed but they show different degrees of distortion (Mueller and Sleumer, 1934).

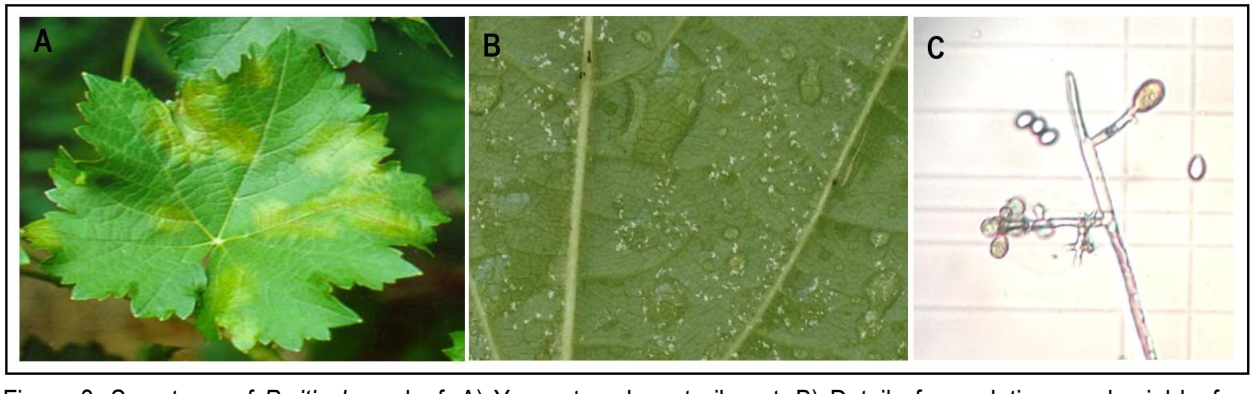

Figure 3: Symptoms of *P.viticola* on leaf. A) Young translucent oil spot; B) Detail of sporulation on abaxial leaf surface after 6 days of inoculum, C) Sporangiophore with attached sporangia, 10x magnification. (picture a by A.Brunelli, pictures b and c by IreneM.Nanni)

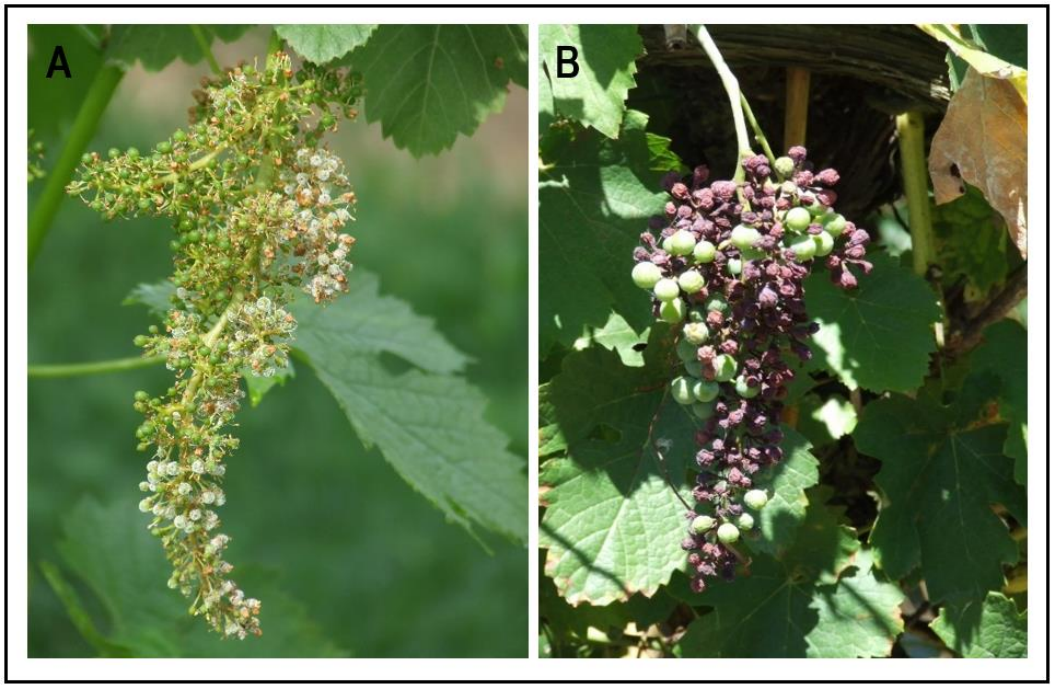

Figure 4: Symptoms on grape. A) Infected inflorescens and young fruit covered with white fluffy spores, B) Berries infected some weeks after postbloom. (pictures by A.Brunelli)

3.2 Life – Disease Cycle

The pathogen overwinters as oospores in dead leaf lesions and shoots (Fig 5). During rainy periods in the spring, the oospores germinate to produce a sporangium. The sporangium or its zoospores are transported by wind or water to the wet leaves on nearby ground, which they infect through stomata of the lower surface. The mycelium then spreads into the intercellular spaces of the leaf, and when it reaches the substomatal cavity, it forms a cushion of mycelium from sporangiophores, which arises and emerges through the stoma. Early studies suggested that the sporulation of *P.viticola* occurs at night (Muller and Sleumer, 1934) and this was confirmed in 1979 by Brook. The sporangia may be carried by wind or rain to nearby healthy plants, germinate quickly, and produce many zoospores that cause secondary infections that rapidly spread the disease (Fig 6).

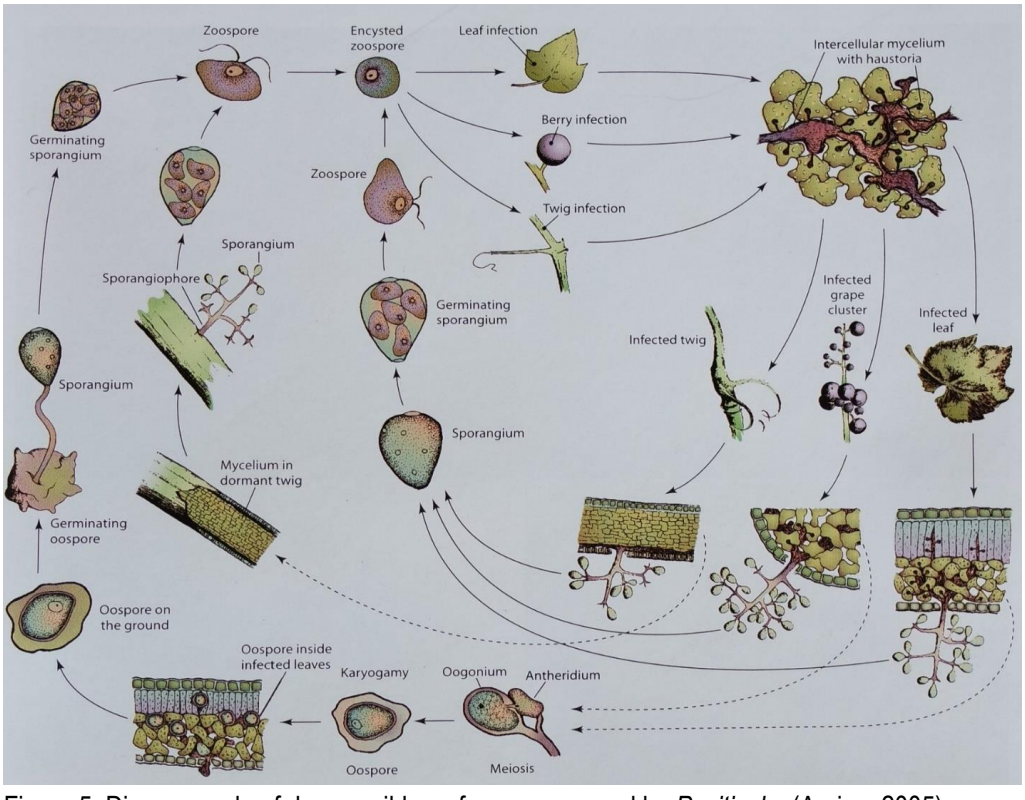

Figure 5*:* Disease cycle of downy mildew of grapes, caused by *P. viticola*. (Agrios, 2005)

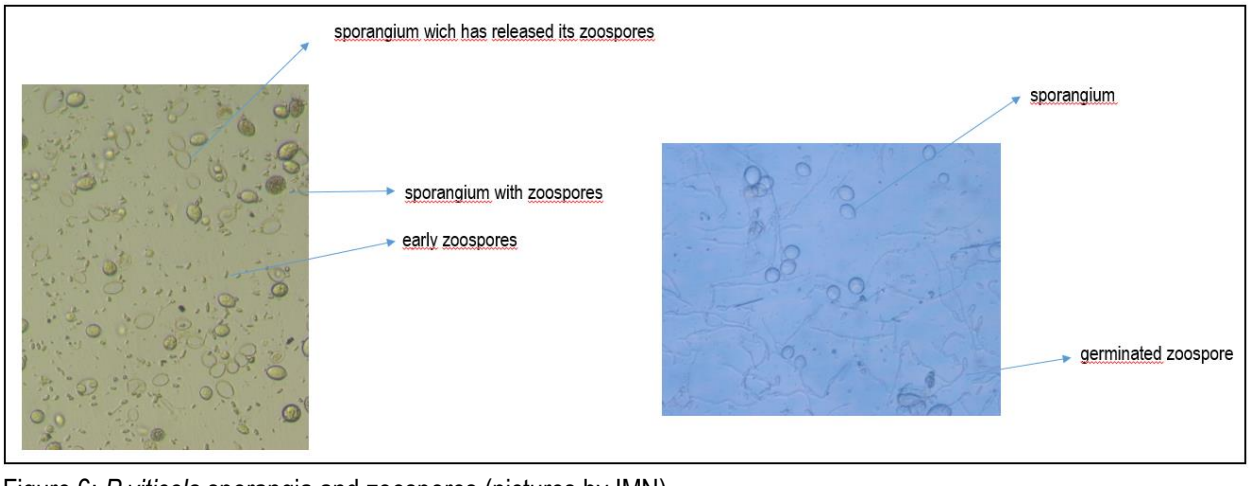

Figure 6: *P.viticola* sporangia and zoospores (pictures by IMN)

4 CELL WALL AND CELLULOSE

4.1 Oomycetes cell wall and cellulose

The cell wall surrounds the cell membrane and determines the morphology of the microorganism. Furthermore, it is involved in the control of exchanges between the cell and the environment. The major contents of oomycetes cell walls are β- (1,3) and β- (1,6)- glucans and cellulose, which have never been reported in any fungal species (Bartinicki-Garcia, 1968). It is possible that the noncellulosic glucan plays a skeletal role in the architecture of these walls. Carbohydrate–active enzymes are involved in the biosynthesis and modification of the cell wall during the life cycle of a microorganism. Many enzymes are involved in these processes, including glycosyltransferases (GTs), glycoside hydrolases and transglycosylase. In addition, carbohydrate-active enzymes, protein kinases and phosphatases may be involved in the morphogenesis of the hyphe. GTs transfer multiple sugar residues to the growing polymer. The products formed by GTs are vital for oomycetes, since they cannot survive without a cell wall (Brown, 2015). As previously stated, cellulose is part of oomycetes cell walls and it is also a major component of plant cell walls. In oomycetes, cellulose type I is present in a poorly crystalline state (Bartinicki-Garcia, 1968). Cellulose is a water–insoluble homopolymer of glucose residues linked by β-(1,4) linkages (Fig 7). The cellulose content varies between oomycetes species, i.e. *Apodachlya* sp (Saprolegniales) contains 4% cellulose (Sietsma,1969), while *Phytium* sp (Peronosporales) contains 20% cellulose (Novaes-Ledieu,1967).

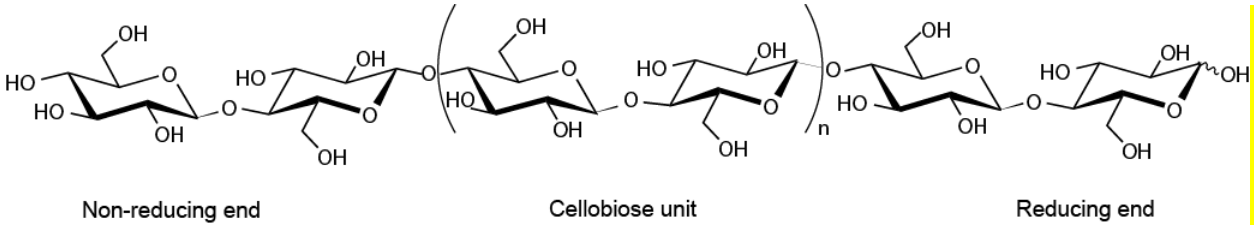

Figure 7: Structure of cellulose chain; the homopolymer is composed by single units of glucose rotated 180° relative to each other forming β - (1,4) linkages. The repeating unit is sometimes considered cellobiose.

Recent work on oomycete cell walls by Melida *et al*. (2013) led to the classification of the two major orders of oomycetes (Peronosporales and Saprolegniales) into three groups based on their cell wall structures. These studies support the existence of *N*- acetylglucosamine (GlcNAc) that contains glucoronic acid and mannose; type II contains up to 5% GlcNAc and residues indicative of cross-links between cellulose and (1,3) - β glucans; type III is characterized by the highest GlcNAc content (> 5%) and the occurrence of unusual carbohydrates that consist of (1,6) - β glucans. The peronosporales are representative of type I cell walls, and the four genera of Saprolegniales (*Achlya, Dictyuchus, Leptolegnia and Saprolegnia*) are classified as type II. Aphanomyces was the only oomycete genus from different organisms studied containing type III cell walls (Melida *et al*; 2013; Brown, 2015).

4.2 Cellulose biosynthesis

Cellulose biosynthesis takes place at the plasma membrane in protein-based complexes, called terminal complexes. In bacteria, tunicates and most algae, these complexes are organized as linear rows of catalytic subunits, single or multiple, depending on the species considered (Brown, 1996). Most of the knowledge about cellulose synthesis comes from studies in bacteria and higher plants (Guerriero *et al*; 2010; Morgan, 2013). Studies on *Arabidopsis thaliana* have shown that the terminal complexes (TCs) are organized as hexamers (rosettes) and each of the six subunits seems to be composed of six cellulose synthase catalytic subunits (Delmer, 1999). However, the proper composition of a rosette is still not clear. It has been demonstrated that the three unique *CesAs* (At*CesA1*, At*CesA3* and At*CesA6*) are needed to form a functional complex for primary cell wall synthesis (Persson, 2007). Along with the *CesA* proteins directly involved in cellulose biosynthesis, there are additional proteins like KORRIGAN, KOBIT01, CSI1 and COBRA. It was also discovered that the cellulose synthesis complex is associated with a sucrose synthase like protein (Fujii *et al*; 2010). Sucrose synthases (SuSy) are known to catalyse the formation of UDP- glucose from sucrose, however the function of this enzyme in cellulose

biosynthesis requires further investigation. As noted in the aforementioned section, there are still many questions to solve to fully understand the mechanism of cellulose biosynthesis in higher plants, yet even less is known about this process in oomycetes. Most of the genes encoding the catalytic subunits of carbohydrate synthases are still not characterized. No *CesA* terminal complex has been observed in oomycetes, as opposed to in plants, algae and bacteria. Yet due to the close phylogenetic relatedness of oomycetes to algae, it is possible to speculate that oomycetes TCs could be organized in single linear rows similar to those noted in some brown algae (Katsaros *et al*; 1996).

4.3 *CesA* proteins

Glycosyltransferases (GT) are classified into distinct families based on enzyme sequences that comprise 92 families of GTs to date. *CesAs* belong to the GT2 family, one of the largest families in the CAZy database (www.cazy.org). Processive GTs belonging to family 2 contain two conserved domains, while most non-processive GTs from the same family contain only domain A, suggesting that the conserved aspartic acid and the QXXRW motif of domain B are involved in the processivity of the enzyme (Fugelstand, 2011). Experiments on the mutagenesis of the two conserved aspartic acid residues in domain A and B, as well as the mutagenesis of the Q, R and W residues in the QXXRW motif in the *CesA* from *Gluconoacetobacter xylinus,* illustrated that these amino acids are necessary for enzyme activity, initiating key research on cellulose synthase associated-genes (Wong *et al*; 1990; Saxena and Brown,1997; Saxena *et al*; 2001). Plant *CesA* genes were discovered for the first time in *Gossypium hirsutum* by Pear *et al.* (1996). In plants, the *CesAs* are predicted to contain up to eight transmembrane (TM) helices, two located at the N-terminal end and the remaining ones at the C-terminus (Somerville, 2006). Eucaryotic *CesAs* have additional domains at their N-terminus; their N-terminal ends include a conserved zinc finger domain (Fig 8). This domain was found to be involved in the homo and hetero dimerization of cotton *CesAs in vitro* and in the yeast two–hybrid system (Kurek *et al*; 2002). Pleckstryn

homology (PH) domain is another example of an N-terminal *CesA* domain and exists only in the oomycetes *CesAs* isoforms1, 2 and 4 (Grenville -Briggs *et al*., 2008 ; Fugelstad *et al*., 2009). This domain seems to be specific to oomycetes and since it occurs in a wide range of proteins involved in signal transduction or as constituents of the cytoskeleton, it was speculated that PH domains in the oomycetes *CesAs* might promote the binding of enzymes to the plasma membrane (Hyvonen and Saraste, 1997).

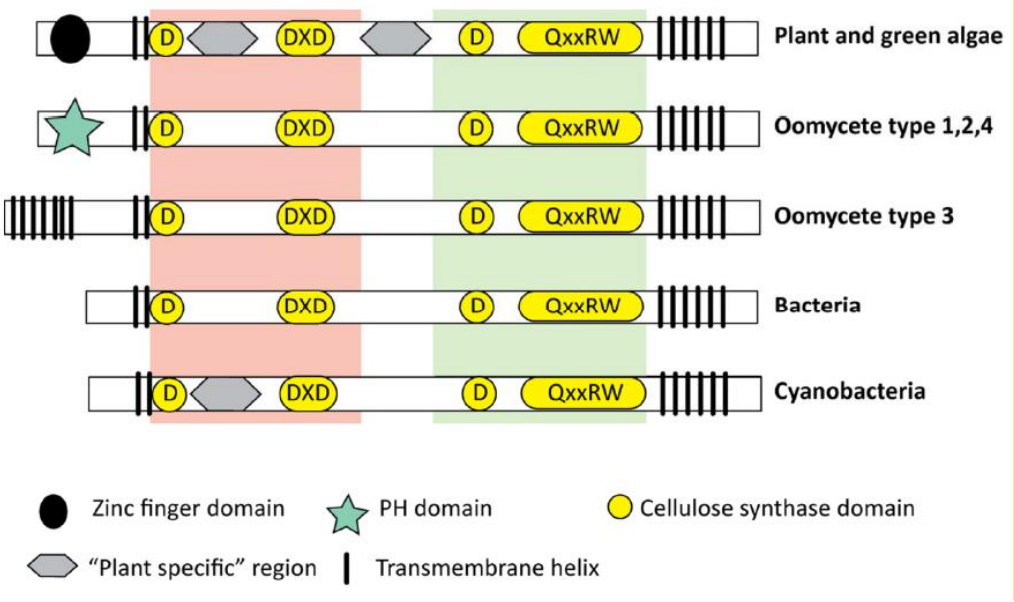

Figure 8: Domain arrangements of *CesAs* from plants, oomycetes and bacteria. In red box: domain A of processive and non-processive GT2s; in green box: domain B of processive GT2s (Fugelstad, 2011).

4.4 *CesA* genes

Despite the economic impact of some oomycetes pathogens like *Plasmopara viticola and Phytophthora infestans,* they are poorly studied and there are few biochemical information available on cellulose synthase enzymes and, consequently, no corresponding gene has been fully characterized to date. The advent of the genomic era enabled the full genome sequencing of some oomycetes species, resulting in the identification of four putative *CesA* genes. This gene family of four cellulose synthase genes was functionally characterized from *P. infestans* utilizing RNA interference, enabling researchers to show

their direct involvement in cellulose biosynthesis (Grenville-Briggs *et al*., 2008). These genes were designated as *CesA1*, *CeSA2*, *CesA3*, and C*esA4*. *CeSA1*, *CeSA2* and *CeSA4* because they share the greatest sequence similarity, but the *CeSA3* was the most divergent member of the family. Expression analysis on *P. infestans* cellulose synthase genes demonstrated that are required for normal appressorium formation *in vitro* and during the germination of cysts. (Grenville Briggs *et al*., 2008). In 2009, Fugelstad *et al* published the identification of the cellulose synthase genes from the oomycete *Saprolegnia monoica,* suggesting their direct involvement in cellulose biosynthesis. Further genes encoding the catalytic subunits of cellulose synthase have also been identified in several bacteria and in plants, but only a limited number of genes that code for proteins involved in carbohydrate synthesis have been documented (Arioli *et al*., 1998, Matthysse *et al*., 1995; Pear *et al*., 1996; Saxena *et al*., 1990).

5 History of anti-Oomycete compounds

The first chemical compound used to control downy mildew was a copper based preparation, called Bordeaux mixture, discovered in 1882 by Millardet. In his work published in 1885, he recommended this treatment to protect grapevines against downy mildew (Millardet *et al*., 1885). Initially, another product called Burgundy mixture was frequently used. This mixture is composed of copper sulfate and sodium carbonate, and was created in 1887 by Masson (Masson *et al.,*1887). Around the same time, some other copper based compounds were produced, by, for instance, Kurtakol (Lustner *et al*., 1922), and Nosperit (Kramer *et al*., 1927). In subsequent years, in order to find an alternative to copper, the fungicidal activities of a number of chemical dyes were tested, including malachite green, brilliant green, rhodainine B, safranin, acridine yellow, auramine and yellow pyocfcanine. Quinosol and sunoxol of the oxyquinoline group of organic substances, were also tested (Meyer, 1932) but none of these substances was ever used in practice. Increased knowledge about the biological cycle of the pathogen enabled the discovery and application of other chemical compounds (acupric fungicides) and, around 1945 after confirmation of greenhouse tests by Du Pont de Nemours the zinc dimethyldithiocarbamate (ziram) was introduced

(Morel, 1946). The subsequent success of acupric fungicides, with multi–site modes of action, was probably related to the development of new stable products, their reduced costs on the market (Zobrist, 1954) and the almost complete absence of phytotoxicity (Gessler *et al.,* 2011). In the 1980s, cytotropic systemic fungicides were discovered, i.e cymoxanil, fosetyl-Al, launched in 1977 (Boubals *et al,* 1979) and metalaxyl (Lafon *et al* ,1978). Since that time, many chemical compounds have been developed and introduced to the market, such as Quinone outside Inhibitor (QoI) that act on mitochondrial complex III on cytochrome bc1 at Qo-site, inhibiting mitochondrial respiration. QoIs are divided into subclasses, such as well known strobilurin (azoxystrobin) and two non-strobilurins, represented by famoxadone and fenamidone. Other fungicides that act on mitochondrial complex III are QiI (Quinone inside inhibitor) i.e. cyazofamid and amisulbrom (Mitani *et al*., 2001). Moreover, QoSI inhibitors like ametoctradin bind to the distal domain of Qo-site (Zhang *et al;* 1998, Fehr *et al*., 2015). Other fungicides introduced to the market have been zoxamide, which acts on the destabilization of microtubule (Young and Slawecki, 2001), fluopicolide, thought to perturb spectrin-like proteins, and carboxylic acid amide (CAAs). To date, according to FRAC, there are nine specific modes of action available for oomycete control.

6 Carboxylic Acid Amide fungicides

6.1 Chemical structures of CAA compounds and their features

The chemical group of carboxylic acid amide (CAA) fungicides was officially announced by the Fungicide Resistance Action Committee in 2005, and they are grouped in the FRAC list with the code 40. The CAA fungicides group includes three subclasses of cinnamic acid amides (dimethomorph, flumorph and pyrimorph), valinamide carbamates (benthiavalicarb, iprovalicarb, valifenalate), and mandelic acid amides (mandipropamid). The reason for this classification was a common cross-resistance pattern amongst all members for the majority of the *Plasmopara viticola* isolates tested (Gisi *et al*., 2012). The mode of action of CAA fungicides was previously associated with an inhibition of phospholipid

biosynthesis, but it has now been confirmed, in studies on mandipropamid, as interference with cell wall deposition and cellulose biosynthesis (Blum *et al*; 2010).

6.2 Cinnamic acid amides

6.2.1 Dimethomorph

Dimethomorph is a cinnamic acid derivative, composed of a mixture of *E* and *Z* isomers, but the fungicidal activity resides exclusively in its *Z* isomer (Albert *et al*., 1988). It shows specific activity against members of the family *Peronosporaceae* and the genus *Phytophthora*. Important diseases controls include *Plasmopara viticola* and *Phytophthora infestans* (Albert *et al*., 1988). Dimethomorph shows translaminar activity following foliar application and exhibits systemic uptake via roots following soil drench. Dimethomorph do not cause phytotoxicity or crop effects even at dose rates well in excess of those required. This compound was introduced onto the market for the first time in 1988 and introduced in Italy in 1994 (Acinapura *et al*., 1992).

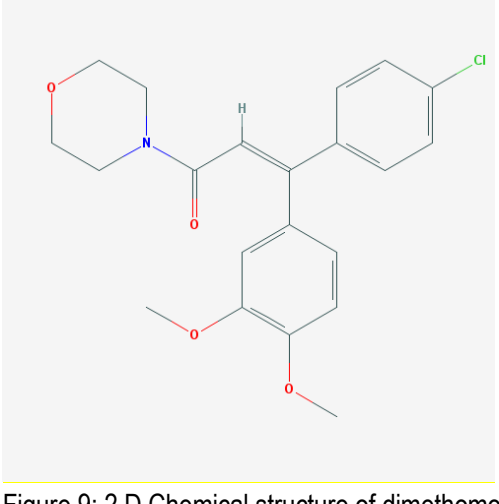

Figure 9: 2 D Chemical structure of dimethomorph, Z- isomer (source pubchem)

6.2.2 Flumorph

Flumorph (Stenzel *et al*., 1998) is a close analog of dimethomorph. The replacement of dimethomorph's chloro substituent by a fluorine atom (see Fig 9 and 10) seems to further improve its antisporulant and curative activities. Flumorph is composed of a mixture of E/Z isomers in a 45:55 ratio and was introduced for the first time in 2000 (Liu *et al*., 2000).

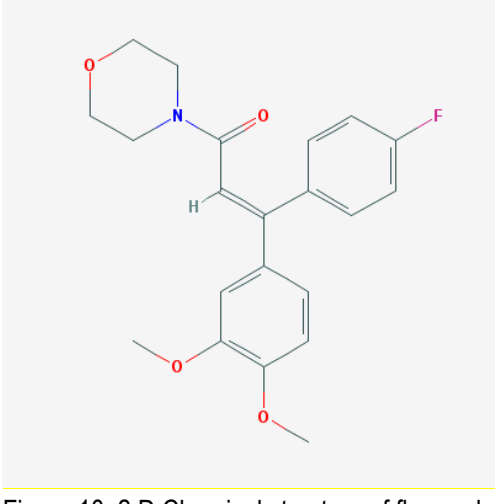

Figure 10: 2 D Chemical structure of flumorph, (source pubchem)

6.2.3 Pyrimorph

Pyrimorph is a novel fungicide that was synthesized by Qin *et al* in (2003) and came on the market in the 2010. This compound exhibits excellent activity against oomycetes, such as *Phytophthora infestans*, *P. capsici* and *Pseudoperonospora cubensis*. Pyrimorph also showed preventive activity, persistence activity, and sporulation inhibition activity against *P. infestans* in glasshouse studies (Chen *et al*., 2007; Huang *et al*., 2007).

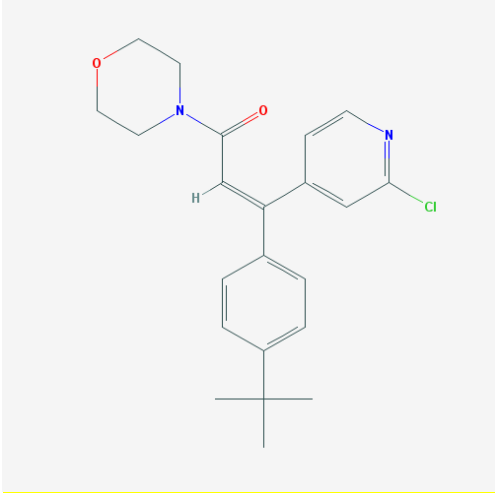

Figure 11: 2 D Chemical structure of pyrimorph, (source pubchem)

6.3 Valinamide carbamates

6.3.1 Benthiavalicarb

Benthiavalicarb–isopropyl was discovered by Kumiai-Ihara, and has been developed for the control of oomycetes diseases. The valinamide derivative benthiavalicarb shows a close structural similarity to iprovalicarb, also containing two chiral centers. Benthiavalicarb was first registered globally in Switzerland and Cuba in 2003, and was introduced in Italy in 2007 (Miyake *et al*.,2003).

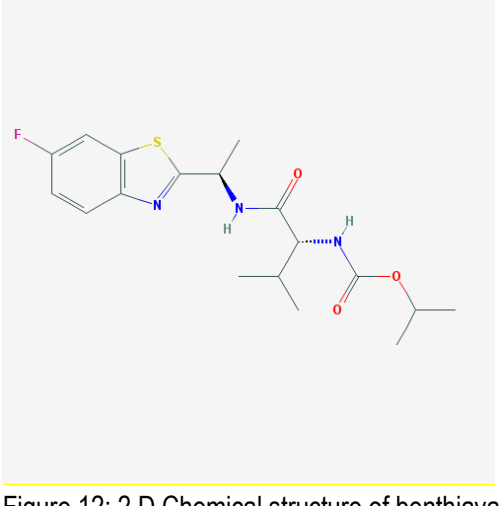

Figure 12: 2 D Chemical structure of benthiavalicarb - isopropyl, (source pubchem)

6.3.2 Iprovalicarb

Iprovalicarb was the first fungicide available on the market from the amino acid amide carbamate class of compounds. It has interesting effects on oomycetes pathogens (Wollweber *et al*., 1990). The iprovalicarb molecule contains two chiral centers; the configuration of the stereocenter in the amino acid function is defined by the use of L-valine as a natural amino acid component, and the active substance contains two diastereomers (the S,S and the S,R- diastereomers). Iprovalicarb was first registered in Indonesia in 1998, the product received approval in Germany in 2000, and has been registered in France and Italy in combination with mancozeb, folpet and with copper. It was approved in Italy in 2002, within a mixture containing mancozeb and fosetyl-Al (Gisi *et al*., 2012).

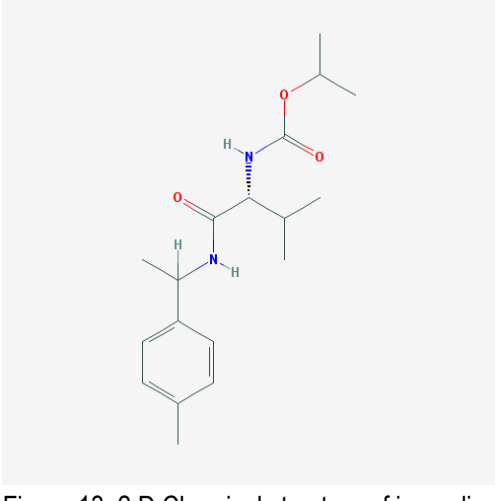

Figure 13: 2 D Chemical structure of iprovalicarb, (source pubchem)

6.3.3 Valifenalate

Valifenalate is a fungicidal dipeptide of the valinamide class of compounds that was launched in Italy and France in 2009 by Isagro (Gonzales-Rodriguez *et al*., 2011).

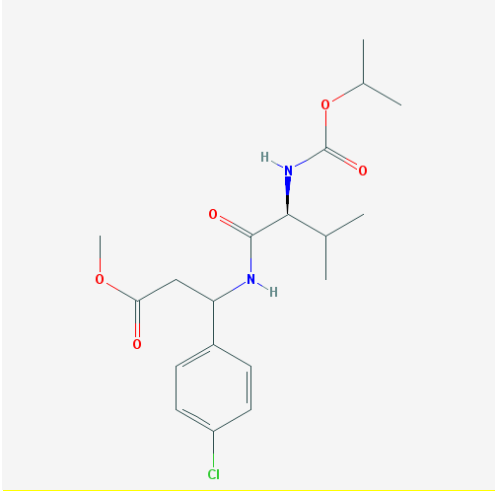

Figure 14: 2 D Chemical structure of valifenalate, (source pubchem)

6.4 Mandelic acid amides

6.4.1 Mandipropamid

The antifungal activity of mandelic acid amides (mandelamides) with dialkoxylated phenethylamine moieties was first discovered for human pathogens by Yu and Van Scott in the mid-1980s. During the early 1990s, the general structure was taken up by chemists at Agrevo (Bayer), who found that the mandipropamid was active against plant pathogens, especially oomycetes (Doeller *et al*.,1994). Then chemists from Novartis (now Syngenta) altered some of the groups of molecules. The exchange of methoxy and ethoxy groups often leads to an increase biological activity. The introduction of a second propargyl group into the mandelic moiety clearly increased its fungicidal activity, finally leading to Syingenta's fungicide mandipropamid. Mandipropamid was first registered in Austria in 2005, and has since been launched in different countries all over the world, both as a solo product and in mixtures (Gisi *et al*., 2012). It was introduced in Italy in 2009.

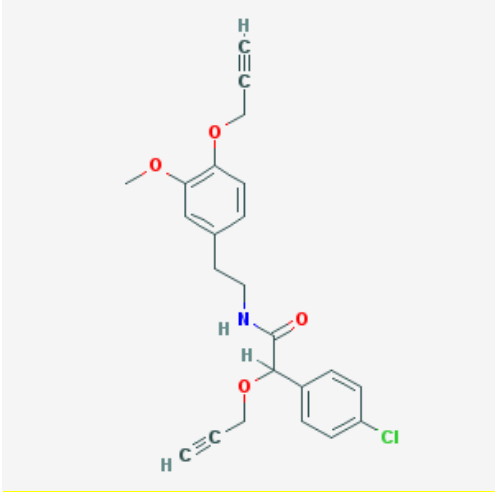

Figure 15: 2 D Chemical structure of mandipropamid, (source pubchem)

7 Fungicide Resistance

Resistance to fungicides is one of the major causes affecting proper disease control. The cause of the development of fungicide resistance is due by multiple factors, among them the extreme adaptability of fungi to their environments. It is to be expected that the evolutionary and dynamic processes of selection would eventually produce fungi that are resistant to fungicides. The rate of resistance development in a population depends on how well characteristics for resistance are inherited, the epidemiology of the fungus, the environment and the persistence of selective pressure, measured as the frequency of fungicide application or the residual properties of the active moiety, or both. The asexual or epidemic growth stage of most plant pathogens is haploid. Consequently, mutational changes are expressed immediately, and, provided the mutant is fit, its development in the fungal population is rapid. The oomycetes fungi are a significant exception because the asexual stage is diploid and the haploid phase is generated during the sexual stage of development. Fungi in which the sexual stage is common readily generate novel genetic forms through the mechanism of recombination, mixing resistance traits with other fitness characteristics and increasing the probability of producing strains that can tolerate applied fungicides. In general, pathogens develop resistance to a fungicide when single or multiple mutations

occur in the genome, resulting in a reduced sensitivity to fungicides. Single-gene mutations conferring resistance to site-specific fungicides occur more frequently than the mutations in multiple genes necessary to confer resistance to multi-site inhibitors. Several mechanisms have been described as conferring resistance to fungicides, such as: alterations and mutations in the target site, mechanisms keeping the intracellular fungicide concentration below a critical threshold included synthesis of efflux transporters that secrete inhibitor molecules to the extracellular compartment, reduced fungicide permeability due to modifications in plasma membranes that can occur, and degradation of the fungicide (Deising *et al*., 2008). Fungicide resistance emerged as a practical problem in the mid-1960s, with the organomercurials on *Pyrenophora avenae*, then continued in 1970 with benzimidazole (thiophanate – metyl and benomyl) in *Venturia nashicola* claimed by Ishii and Yamagutchi in 1977, and on to *Venturia inaequalis* (Koenraadt *et al*., 1992). Several other classes of fungicides, such as pyrimidines, phenylamides, dicarboximides, DMIs, carboxanilides, morpholine and strobilurins, developed resistance in over time (Table 2). The degree of response of fungal populations to fungicides used for their control splits products into the following categories: low resistance-risk fungicides, high resistance-risk fungicides and moderate resistance-risk fungicides (Hewitt, 1998).

Table 2 : Occurrence of resistance in Northern Europe against fungicide of different classes; modified after Hewitt (Deising *et al*, 2008)

Fungicide class	**Number of targets**	**First report of resistance**	**Pathogen involved**
organomercurials	many	1964	*Pyrenophora avenae*
benzimidazoles	1	1970	*Venturia inaequalis, Venturia nashicola*
phenylamides	1	1980	*Plasmopara viticola, Phytophtora infestans*
dicarboxamides	1	1982	*Botrytis cinerea*
DMIs	1	1982	*Blumeria graminis*
carboxanilides	1	1986	*Ustilago nuda*
morpholines	2	1994	*Blumeria graminis*
strobilurins	1	1998	*Blumeria graminis*

7.1 CAAs resistance risk

Compounds with a site-specific mode of action, such as CAA, are more prone to develop resistance than multi-site ones, and the resistance is also associated to the biology of the pathogen involved. A recent study on mandipropamid fungicide claimed that the inheritance of resistance of this compound, in the diploid organism *P.viticola,* was recessive and associated with one or two nuclear genes (Gisi *et al*., 2007). Single amino acid exchanges in *CesA3* have been detected, conferring resistance to CAA fungicides in different plant pathogens of the *Peronosporales*, such as *P. infestans* (Blum *et al*., 2010a), *Phytophthora capsici*, *Phytophthora melonis* (Chen *et al*., 2012), *P.viticola* (Blum *et al*., 2010b) and *Pseudoperonospora cubensis* (Blum *et al*., 2011). In most cases, resistance was based on a single point of mutation in the *CesA3* gene, leading to a change of amino acid position 1105 from a conserved glycine to either alanine, serine, valine or tryptophan (G1105A, G1105S, G1105V, G1105W) (Blum *et al.,* 2010a , Blum *et al*., 2010b, Blum *et al*., 2011, Sierotzki *et al*., 2011. Chen *et al* (2012) claimed that a change from valine to leucine or methionine at position 1109 in artificial mutants also confers resistance to CAAs. Resistance to CAAs in *P.viticola* field populations has been reported in France and Germany for almost 20 years, in particular resistance to dimethomorph, first discovered in France in 1994 (Chabane *et al*.,1996), but it hasn't created any problems for growers. In *Phytophthora* species (mainly *P. infestans*), resistance to CAAs has never been detected in field populations, even though dimethomorph has been used for more than 10 years (Gisi and Sierotzki*,* 2008). CAA resistant isolates were also reported in *Pseudoperonospora cubensis* populations (Zhu *et al*., 2007, Blum *et al*; 2011). Furthermore, Wang *et al.* (2010) showed that *Peronophythora litchii* resistant isolates were able to express resistance to CAAs (dimethomorph, flumorph, mandipropamid, pyrimorph) under laboratory conditions. In the downy mildew pathogen *P.viticola*, some resistant field isolates have been detected for the first time in France (Chabane *et al*; 1996) followed by other resistant phenomenon from different European countries (Gisi et al; 2007). To increase the knowledge about the practical resistance in field

toward CAAs, in chapter nine, we provide the first evidence about the presence of mandipropamid resistant populations of *P. viticola* from commercial vineyards in Italy.

8 Screening of sensitivity to mandipropamid of *Plasmopara viticola* populations from Italian vineyards by molecular and biological methods

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Abstract

Aims: The main object of this work was to study the sensitivity to mandipropamid, a carboxylic acid amide (CAA) fungicide introduced in Italy in 2009, of thirty-three *Plasmopara viticola* populations utilizing both molecular and biological techniques. The PCR-RFLP technique was developed in order to detect the single point mutation G1105S occurring on the Pv*CesA3* gene. The sensitivity was also studied using the leaf-disc bioassay. Methods and Results: Thirty-three downy mildew-infected leaf samples, collected from 2010 to 2013 from Italian vineyards located in Italy, were used in the study. PCR-RFLP revealed the presence of twelve sensitive profiles, fourteen mixed (sensitive and resistant) and seven resistant profiles. Half-maximal inhibitory concentrations (EC_{50}) calculated from the bioassays showed an EC_{50} < 1 mg l^{-1} for samples that showed sensitive profiles, while for those samples that had a mixed profile, EC_{50} ranged from <1 to >300 mg l^{-1}, and values for resistant profiles ranged from 200.28 to >300 mg l^{-1}. Conclusions: The results suggest that *P. viticola* populations infecting Italian vineyards are under a selection pressure due to CAA based fungicide applications. This information should be considered in order to improve the current grapevine downy mildew management strategies and to minimize the risk of the development and spread of CAA-resistant populations. Significance and Impact of the Study: In this work, we provide the first evidence about the presence of mandipropamid resistant populations of *P. viticola* from commercial vineyards in Italy.

Keywords: carboxylic acid amides, cellulose synthase, grape downy mildew, point mutation, -PCR-RFLP, G1105S

Introduction

Grape downy mildew caused by the oomycete *Plasmopara viticola* (Berk. & Curt) Berl. & de Toni is one of the most devastating diseases affecting grapevine (*Vitis vinifera* L.) worldwide (Emmett *et al*.1992). As the pathogen attacks both leaves and berries, the disease represents a serious limitation to grapevine production.

In Italy, as in other countries, disease management consists in the application of chemical fungicides. Beside protectant fungicides such as dithiocarbamates, dithianon and folpet, which have a multi-site activity, many modern fungicides with a single-site activity and different modes of action have been introduced to control this disease. These molecules belong to several chemical classes such as phenylamides (metalaxyl-M, benelaxyl-M), acetamides (cymoxanil), QoI (strobilurins, famoxadone, fenamidone), QiI (cyazofamid) and more recently QoSI (ametoctradin). Because of the single-site activity of modern fungicides, the occurrence of fungicide resistance is a risk. In fact, *P. viticola* is considered to be a high risk pathogen regarding the acquisition of fungicide resistance (Fungicide Resistance Action Committee, FRAC) as demonstrated by the occurrence of resistance to QoI fungicides in Italian and French populations (Genet *et al*.2006) and in other European countries (Sierotzky *et a*l.2011). Current resistance management strategies include limitation of the number of applications, use of mixtures and alternation of fungicides with different modes of action. The recently introduced chemical classes include Carboxylic Acid Amide (CAA) fungicides. CAA represent compounds from three different chemical groups; cinnamic acid amides, valinamide carbamate and mandelic acid amide as officially reported by FRAC in 2005. Dimethomorph was the first CAA fungicide used to control downy mildews (Albert *et al*.1988). In Italy, it has been authorized on grape since 1994 while the other CAA compounds were introduced in the early 2000s: iprovalicarb in 2002, benthiavalicarb in 2007, valifenalate and mandipropamid in 2009. Biochemical studies have demonstrated that CAA fungicides inhibit processes involved in cell wall biosynthesis (Jende *et al*.2001), and in particular, as recently elucidated by Blum et al. (2010a), in the inhibition of cellulose biosynthesis. In oomycetes, up to four cellulose synthase (*CesA*)

genes have been identified and their direct involvement in cellulose biosynthesis has been proved utilizing RNA interference in *Phytophthora infestans* (Grenville-Briggs *et al*.2008); a recent study also reported that the four genes are upregulated during cyst germination and appressoria formation in *P. infestan*s (Grenville-Briggs *et al*.2008). Single amino acid exchanges in *CesA3* protein conferring mandipropamid resistance in *P. viticola* have been identified. The G1105S mutation leading to an amino acid change from glycine to serine at codon 1105 was detected in 2011 (Blum *et al*.2010b). Moreover, G1105V mutation leading to an amino acid change from glycine to valine at the same codon 1105 was detected by Sierotzky *et al*. (2011). The recessive nature of CAA resistance led the FRAC group to classify the resistance risk as moderate, and cross-resistance among its members was clearly demonstrated (Gisi *et al*.2007). The occurrence of CAA fungicide-resistant populations of *P. viticola* was described for the first time in France in 1994 (Chabane *et al*.1996) for dimethomorph. During the following years, resistance increased gradually in Europe (Sierotzki *et al*. 2011; FRAC). Studies on sensitivity to CAA and in particular to mandipropamid were carried out on sensitive *P. viticola* Japanese populations by Aoki *et al*. (2011), utilizing a PCR-RFLP based method that easily allowed identification and discrimination of sensitive and resistant *P. viticola* isolates.

The aim of this work was to screen the sensitivity to mandipropamid of *P. viticola* populations collected from vineyards in Italy. To reach this goal, both PCR-RFLP and the leaf disc bioassays (the conventional method to test obligate pathogens according to FRAC) were adopted.

Material and Methods

Sample collections

Leaf samples showing typical symptoms of downy mildew, oil spots and abundant sporulation on the abaxial and adaxial leaf surfaces respectively were randomly collected from thirty-three commercial vineyards located in Northern Italy between 2010 and 2013 (Table 1). From twenty-five to thirty leaves

per field were collected from each vineyard and considered to be representative of *P. viticola* populations. Both biological and molecular assays were carried out for each population.

Table 1 Geographical origin and year of collection of *P.viticola* populations

Population	Location[a]	Year of collection
236	Gorizia (FVG)	2010
237	Gorizia (FVG)	2010
238	Gorizia (FVG)	2010
239	Pordenone (FVG)	2010
240	Pordenone (FVG)	2010
241	Bologna (ER)	2010
242	Bologna (ER)	2010
247	Treviso (VEN)	2011
253	Asti (PDM)	2012
255	Trento (TAA)	2012
260	Trento (TAA)	2012
264	Trento (TAA)	2012
266	Trento (TAA)	2012
268	Modena (ER)	2012
269	Modena(ER)	2012
CN	Cuneo (PDM)	2012
RA	Ravenna (ER)	2012
272	Trento (TAA)	2013

275	Trento (TAA)	2013
276	Trento (TAA)	2013
277	Trento (TAA)	2013
279	Trento (TAA)	2013
280	Bologna (ER)	2013
281	Ancona (MAR)	2013
284	Cuneo (PDM)	2013
285	Pescara (ABR)	2013
286	Firenze (TUS)	2013
287	Modena (ER)	2013
289	Modena (ER)	2013
290	Modena (ER)	2013
291	Modena (ER)	2013
292	Modena (ER)	2013
G	Bologna (ER)	2013

[a] Abbreviations indicate the corresponding region where samples were collected:
FVG: Friuli Venezia Giulia
ER: Emilia Romagna
PDM: Piedmont
TAA: Trentino Alto Adige
MAR: Marche
ABR: Abruzzo
TUS: Tuscany
VEN: Veneto

Biological assays

In order to test the sensitivity of *P. viticola* populations to mandipropamid, leaf disc bioassays were carried out using the method described by Wong and Wilcox (2000) with some modifications. Collected

leaf samples were kept in plastic bags and transferred to the laboratory to be directly processed. Mandipropamid (Pergado SC, Syngenta Italia, Milan, Italy) was tested at six concentrations: 1, 3, 10, 30, 100, 300 mg l^{-1}.

Leaf discs (22 mm ∅) were obtained from cv Chardonnay grapevine leaves and soaked in each product concentration. Distilled water was used for the untreated control. After forty minutes, leaf discs were removed, dried on a grate and then transferred to Petri dishes containing agar medium (1.5%) complemented with 2 mg l^{-1} of kinetin. Each concentration consisted of three replicates, each one represented by a Petri dish containing five leaf discs and bioassays were repeated three times. The sporangial suspension was obtained from twenty-five to thirty leaves collected randomly from each vineyard and considered to be representative of the vineyard population. The concentration was verified utilizing a hemocytometer, and the inoculation was carried out 24 h after treatment by spraying the sporangial suspension (5×10^4 spores ml^{-1}) onto the adaxial surface of each disc. Petri dishes were then incubated at 20°C temperature with a 12 h of photoperiod. Disease leaf area was assessed 8-10 days after inoculation by evaluating the percentage of sporulation at the stereo microscope. Data were then used to calculate the half maximal effective concentration (EC_{50} in mg l^{-1}) using probit analysis. According to Gisi *et al*. (2007), samples were considered to be sensitive to mandipropamid when EC_{50} was between 0.03 to 3.6 mg l^{-1}.

DNA extraction and PCR-RFLP analysis

DNA extraction of the 33 samples was performed from the same sporangial material (5×10^4 spores ml^{-1}) used for bioassays using the cetyltrimethylammonium bromide (CTAB) method following the protocol of Doyle and Doyle (1987) with some modifications. Sporangial suspension was collected as a pellet by centrifuging at 14,000 rpm for 20 min. The CTAB solution was first heated at 65°C for 1 h and 500 µl was then added to the pellet. 2.5 µl of proteinase K (10 mg l^{-1}) was added to the CTAB solution. The solution was heated at 65°C for 30 min in the water bath. Subsequently, 500 µl of a mixture of

chloroform-octanol (24:1) was added to the solution and centrifuged for 5 min at 8000 rpm. After the addition of 5 µl of RNAase (10 mg l^{-1}) to the supernatant, 1 ml of chloroform-octanol was added and the solution was centrifuged as described above. DNA precipitation was carried out by adding 0.7-0.8 volumes of isopropanol to the supernatant and centrifuging for 20 min at 14000 rpm. The pellet was then dried under vacuum, washed with 500 µl of 70% ethanol and centrifuged for 5 min at 12000 rpm. Finally, the pellet was air dried and re-suspended in 20-50 µl of MilliQ water.

A Pv*CesA3* gene fragment including the region codifying the G1105S mutation was amplified by using the forward primer Pv*CesA3*F (Blum et al. 2010b) and a newly designed reverse primer Pv*CesA3*R (Table 2). PCR amplifications were performed in a final volume of 25 µl using 10× *Ex Taq* Buffer, 2.5 mM of each dNTP, 10 µM of each primer, 1 µl of total DNA (20 ng/µl) and 0.75 units of *Ex Taq* DNA polymerase (Takara, Shiga, Japan). The reaction started at 94°C for 4 min and continued for 35 cycles at 94°C for 30 s, 50°C for 30 s 72°C for 20 s, and the final extension was at 72°C for 7 min. From the PCR product, 5 µl were then digested with 0.25 U of *Pvu*II restriction enzyme (Promega, Madison, WI, USA) which, similarly to that used by Aoki et al. (2011), is able to recognise its target site only when the mutation causing G1105S substitution is present. After electrophoresis, the polyacrylamide gel (6.7%) was stained with ethidium bromide and visualised under UV light. The size of DNA fragments were determined by comparison with the marker pBR322 (New England BioLabs, Beverly, MA, USA).

Table 2 Primer sequences selected for PCR-RFLP analysis.

Name	(5'→3')	Sequence	Amplicon (bp)
Pv*CesA3*F	Forward	ACCCCATGGTCAAGATGAGTATC	195
Pv*CesA3*R	Reverse	GGCAATGCCGAAACTGGGGATG	

Results

In this study, both the PCR-RFLP method and bioassays were carried out to rapidly screen the sensitivity of *P. viticola* to mandipropamid.

Biological assays showed a wide range of EC_{50} values among *P. viticola* populations. In particular, populations from Friuli Venezia Giulia (FVG) vineyards showed EC_{50} values ranging from 2.75 to 106.69 mg L^{-1}, while those from Piedmont (PDM), Emilia Romagna (ER), Veneto (VEN) and Trentino Alto Adige (TAA) vineyards showed EC_{50} values ranging from < 1 to > 300 mg L^{-1}. Three populations from the Marche (MAR), Tuscany (TUS) and Abruzzo (ABR) regions showed EC_{50} < 1 mg L^{-1} (Table 3).

The PCR-RFLP method was used to detect a single point mutation G1105S that occurs in the Pv*CesA3* gene, utilizing the forward primer *Pv*CesA3F (Blum et al. 2010b) with a newly designed reverse primer Pv*CesA3*R and utilizing the *Pvu*II restriction enzyme. The technique that we applied in this study gave us the possibility to screen the sensitive, the resistant and the mixed (R/S) populations. Both sensitive populations with a 195 bp band and resistant populations with two bands of 116 bp and 79 bp were identified. Selected amplicons were then sequenced to confirm either the absence or the presence of the G1105S substitution in the sensitive and resistant profiles, respectively (GenBank Accession Numbers KM066600 and KM066601).

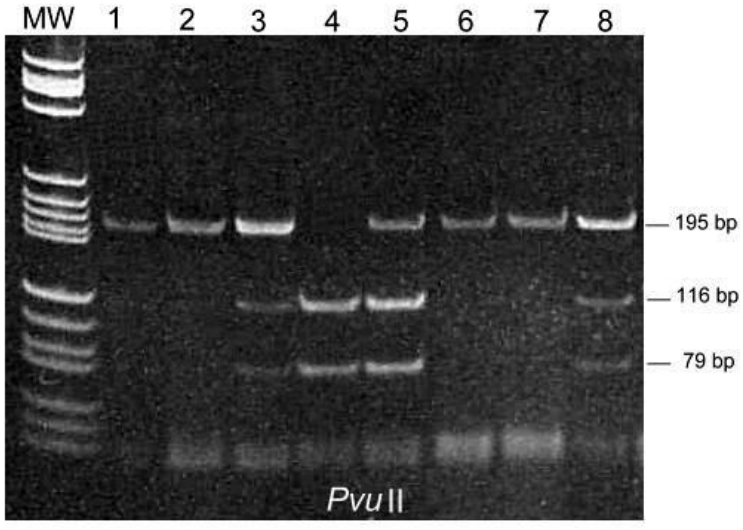

Fig. 1. PCR-RFLP analysis with *Pvu*II (primers Pv*CesA3*F and Pv*CesA3*R). Lanes: MW, DNA molecular weight marker pBR322; sensitive populations (1, 2, 6, 7); resistant population (4); R/S (mixed populations) (3, 5, 8).

Fourteen of the thirty-three populations examined contained a mixture of resistant and sensitive isolates as indicated by their PCR-RFLP profiles. In particular, in all Friuli Venezia Giulia populations a mixed profile pattern was detected showing three bands at 195, 116 and 79 bp (Fig.1). The same pattern was also obtained in the populations from Marche and Veneto and in six populations from Emilia Romagna and one from Trentino Alto Adige (Table 3). Moreover, five populations from Trentino Alto Adige were considered to be resistant as they showed two bands at 116 and 79 bp. The R pattern was also identified in two samples from Piedmont, while sensitive populations were identified from Emilia Romagna (six populations), Trentino Alto Adige (three populations) and Piedmont, Tuscany, and Abruzzo (one population each).

All samples with EC50 > 1 mg l^{-1} showed either the R or the R/S profile in the PCR-RFLP analysis. In particular, all samples classified by R had high EC_{50} values (> 200 mg l^{-1}) while, in general, populations that showed a mixed profile had a wide range of EC_{50} values, ranging from <1 to > 300 mg l^{-1}.With the exception of population 281, that showed an R/S profile, all samples with the S profile had an EC_{50} <1 mg l^{-1}.

Table 3 EC_{50} values and PCR-RFLP profiles of *Plasmopara viticola* populations

Population	Geographical origin[a]	EC_{50}	PCR-RFLP profile
236	FVG	17.32	R/S[b]
237	FVG	3.96	R/S
238	FVG	2.75	R/S
239	FVG	7.42	R/S
240	FVG	106.69	R/S
241	ER	5.82	R/S
242	ER	1.98	R/S
247	VEN	4.11	R/S
253	PDM	>300	R
255	TAA	<1	S
260	TAA	<1	S
264	TAA	>300	R/S
266	TAA	<1	S
268	ER	<1	S
269	ER	<1	S
CN	PDM	<1	S
RA	ER	<1	S
272	TAA	>300	R
275	TAA	>300	R
276	TAA	200.28	R
277	TAA	>300	R

279	TAA	>300	R
280	ER	<1	S
281	MAR	<1	R/S
284	PDM	>300	R
285	ABR	<1	S
286	TUS	<1	S
287	ER	5.66	R/S
289	ER	<1	S
290	ER	10.54	R/S
291	ER	38.64	R/S
292	ER	>300	R/S
G	ER	<1	S

[a] For the legend see table 1
[b] R/S: mixed; R: resistant; S: sensitive

Discussion

This study evaluated the sensitivity of Italian *P. viticola* populations to mandipropamid, a member of the carboxylic acid amide fungicides. Both molecular and biological methodologies were applied for this screening, in order to have a more accurate and rapid detection of both sensitive and resistant populations coming directly from the field. Moderate EC_{50} values can be explained by populations containing a mix of sensitive and resistant strains. In the leaf tests, only the resistant isolates survived and sporulated, leading to the increase of EC_{50} values. Moreover, when the majority of the population is resistant, leaf disc tests showed high infection values and therefore high EC_{50} values. This is the case of the populations classified as R and most probably also the populations having R/S profiles with EC_{50} >300 mg l^{-1}. The 281 population with a mixed profile (R/S) was phenotypically sensitive; in fact, only the

sensitive gave sporulation in the leaf disc assay, while the PCR-RFLP method was also able to detect the not-surviving and the alive strain, which could explain the detection of the R/S profile with an EC_{50} <1 mg l^{-1}.A rapid detection method is often necessary to better understand the incidence of the resistance to fungicides of *P.viticola* in Italian vineyards. Conventional fungicide sensitivity assays for obligate pathogens should be performed *in vivo,* i.e. disc leaf assays and whole plants, and are time consuming. PCR-RFLP, on the other hand, is considered to be a rapid tool and requires approximately 4/5h for the detection of the mutation (Aoki *et al.* 2011). In this study, PCR-RFLP was able to detect sensitive, resistant and mixed profiles (R/S) among the populations. All populations tested with the PCR-RFLP method were also evaluated by the *in vivo* bioassays, because as mentioned before this is the conventional method used to test obligate pathogens according to FRAC. Albeit with a different restriction enzyme, the PCR-RFLP method had already been used by Aoki *et al*.(2011) to identify the G1105S mutation in the Pv*CesA3* gene and, similarly to Aoki *et al*. (2011), this method was easily reproducible to rapidly screen the presence of the G1105S mutation involved in the mandipropamid resistance phenomenon. Unlike that study where the resistant allele was synthetic, in our screening we found the resistant allele in natural populations. This suggests that *P. viticola* populations infecting Italian vineyards are under a selection pressure due to mandipropamid applications. This information should be taken into consideration in order to improve the current grapevine downy mildew management strategies and to minimize the risk of the development and spread of mandipropamid and other CAA resistant populations. Based on these findings, since the nature of this resistance gene Pv*CesA3* is recessive (Gisi et al., 2007), improving our knowledge about the resistant population development of a quantitative molecular method that quantifies the percentage of mutated alleles in the Pv*CesA3* gene represents an essential step in further evaluating *P. viticola* sensitivity to CAA fungicides.

Acknowledgements

The authors wish to thank Dr. Gerd Stammler (BASF, Agricultural Research Centre - Limburgerhof, Germany) for helpful comments on this manuscript.

Conflict of Interest

No conflict of interest declared

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9 Differences in the efficacy of Carboxylic Acid Amides (CAA) fungicides against less sensitive strains of *Plasmopara viticola*

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Abstract

Plasmopara viticola is controlled by fungicides with different modes of action, including carboxylic acid amides (CAAs). The aim of this study was to evaluate differences of CAA resistant *P.viticola* strains towards CAAs. The results show that the G1105S mutation affect all four CAAs, but with different impacts. While this confirms that they have the same mode of action, it shows that differences between CAAs can occur. Further molecular modelling and docking studies are needed to better understand the different behaviors reported here.

Keywords: Oomycetes; *CesA3* gene; cellulose biosynthesis; resistance

This paper has been published in Pest Management Science (DOI10.1002/ps.4182) and it has been obtained the license to reprint it (license n: 3838711321493).

Introduction

Downy mildew caused by *Plasmopara viticola* (Berk. & Curt) Berl. & de Toni (Figure 1), is a disease affecting grapes worldwide. Carboxylic acid amides (CAAs) are widely used for control of this pathogen. Dimethomorph was the first CAA introduced in 1988, followed by iprovalicarb in 1998, flumorph in 2000, benthiavalicarb in 2003, mandipropamid in 2005, valifenalate in 2008 and pyrimorph in 2010 (Kraemer *et al*., 2012). The mode of action is linked to the inhibition of the cellulose biosynthesis that is regulated by a family of four *CesA* genes (Blum *et al*., 2010). Mutations conferring CAA resistance are G1105S and G1105V in the *CesA3* gene (Sierotzky *et al*., 2011) CAA resistance is inherited in a recessive manner (Gisi *et al*.,2007)

The aim of this report is to present differences in the response of CAA resistant *P. viticola* strains towards different CAAs. Samples were collected from different Italian vineyards and tested for their sensitivity towards dimethomorph and mandipropamid. The preventive and curative activity of benthiavalicarb, dimethomorph, iprovalicarb and mandipropamid was tested in separate assays with a CAA sensitive and resistant strain.

Experimental methods

P. viticola samples and strains

Leaf samples showing typical symptoms of downy mildew collected from 42 commercial vineyards in Northern Italy in 2014 were utilized to generate the 42 *P. viticola* populations used in leaf disc tests to determine EC_{95} values. Detached leaf tests were conducted with a CAA sensitive strain isolated in Germany in 1999 and a resistant strain (with 100% G1105S mutation) isolated in France in 2002.

Bioassays on leaf discs

Bioassays were carried out utilizing grape leaf discs (cv. Chardonnay) applying 6 concentrations (1, 3, 10, 30, 100, 300 mg L^{-1} a.i.) of mandipropamid (Pergado SC) and dimethomorph (Forum 50 WP) 24 hours before inoculation. For each concentration tested there was an untreated control included, 15 leaf

discs were soaked in the fungicide suspensions for each concentration for 45 min. The inoculation was conducted by spraying a sporangial suspension ($5x10^4$ spores mL^{-1}) onto the adaxial surface of leaf discs which were then incubated at 23°C and 12 hours of photoperiod.

The sporulation was assessed 8-10 days after the inoculation by evaluating the % of sporulated leaf area and the EC95 values (mg L^{-1}) were calculated by probit analysis.

Detached leaf tests

Leaves from 10-week-old greenhouse plants were cut off and placed upside down in Petri dishes with water agar (0.4 % agar + 40 mg L^{-1} benzimidazole + 30 mg L^{-1} streptomycin) and sprayed with benthiavalicarb, dimethomorph, iprovalicarb and mandipropamid (all tech a.i., from Sigma Aldrich, St. Louis, MO, USA) solved in a standard formulation containing 5% acetone and 0.05% Wettol LF700. Rates of a.i. were chosen based on their use in combination products (Anonymous, 2015), where the maximum registered field rates of the solo a.i.s in combination products are 225 g ha^{-1} a.i. dimethomorph, 150 g ha^{-1} a.i. mandipropamid, 220 g ha^{-1} a.i. iprovalicarb and 35 g ha^{-1} a.i. benthiavalicarb, respectively. Leaves were sprayed to just before run off, which was then calculated with 1000 L ha^{-1}, resulting in concentrations of 225, 150, 220 and 35 mg L^{-1}, respectively. Additionally, half rates were used. For preventive trials, application was 1 day before inoculation; for curative trials, 1 day after inoculation. Inoculation was done with a suspension containing $2x10^5$ sporangia mL with an airbrush and around 500 µl suspension per leaf. Four inoculated leaves (each leaf in one Petri dish) were used as replicates for each strain (CAA sensitive and resistant). Petri dishes were incubated for 18 – 20 h in darkness in a moist chamber at 18°C. The Petri dish lids were then removed and the surfaces of the leaves dried in the horizontal laminar flow cabinet. The lids were then replaced and the Petri dishes further incubated at 20°C with 12 h light/darkness. After 6 days, the inner surfaces of the Petri dish lids and the surfaces of the leaf discs were sprayed with distilled water to stimulate sporulation and incubated for a further 24 h. Seven days after the inoculation the % infected (sporulating) area of each

leaf was assessed. Efficacy was calculated based on the four replicates (leaves): ([% diseased leaf area untreated - % diseased leaf area treated]/% diseased leaf area untreated) x 100%.

Results and discussion

The CAA compounds tested on 42 populations coming from commercial vineyards with bioassays on leaf discs showed different level of efficacy, generally dimethomorph showed lower EC_{95} values compared to mandipropamid (Figure 1).

Detached leaf tests showed that under preventive conditions the CAA-sensitive strain was fully controlled by all CAAs at both rates. The resistant strain was controlled at different levels by the CAAs under preventive conditions. Dimethomorph and mandipropamid showed the highest levels of activity followed by benthiavalicarb and iprovalicarb (Figure 2). The tests confirmed that all CAAs have a curative activity against the CAA-sensitive strain. However, this curative activity is lost on the CAA resistant strain. This difference in preventive and curative control of the CAA-resistant strain appears to be linked to the mode of action of CAAs. Albert et al. (1991) have described that downy mildew fungi are most sensitive to CAAs when cell walls are being actively synthesized during zoospore encystment, zoospore germination and mycelial development stages. In the preventive tests, the a.i.s can take advantage of this by stopping fungal development very early before any fungal mass develops. This activity window is lost in the curative test especially under the test conditions, which were set up for a rapid establishment of the infection and fungal development. Such a rapid fungal development in the field is rather unlikely, because of less optimal conditions (e.g. temperature, humidity) and it is currently not known how CAA performance decreases on CAA resistant strains under practical field conditions, where the establishment and infection progress is slower than under our test conditions. There might be still some residual curative activity also on CAA resistant strains, especially when application is close to the infection time point.

In our preventive trial set up the tested CAAs controlled the resistant strain differently. However, the data also confirm the cross resistance between CAAs, especially due to the fact that no CAA controlled the resistant isolate under curative conditions. Our observation that a target site mutation may have a different impact on individual CAAs is also known for other mode of action groups such as QoI (Pasche *et al*., 2005), SDHI (Semar *et al*.,2013) and DMI (Cools *et al*., 2013). This may be based in the different structure of the molecules, but molecular modelling and docking studies could so far not explain the different behavior because of uncertainties on the binding site of CAAs at the *CesA3* protein (data not shown). Further modelling and related studies are needed to understand the differences reported here among CAA fungicides.

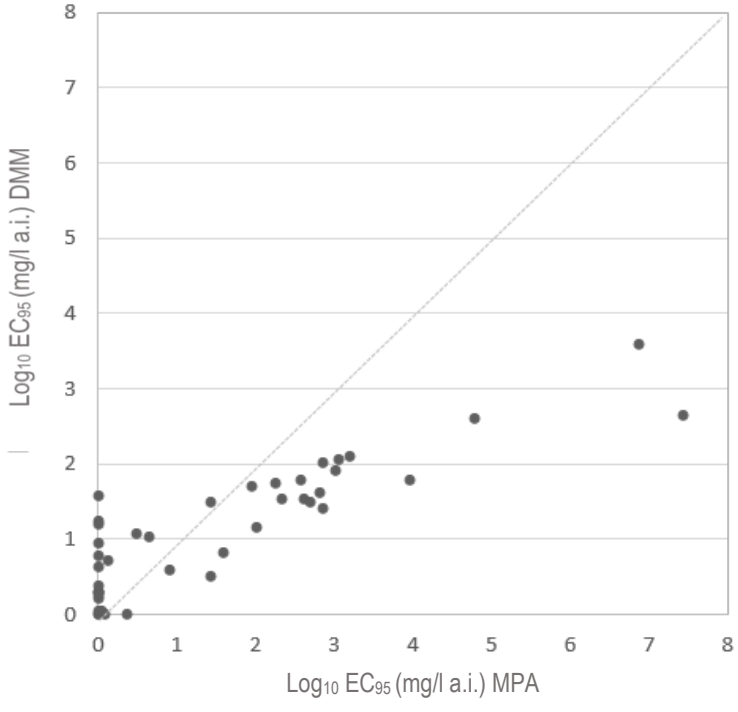

Figure 1 Distribution of the sensitivity (Log_{10} EC_{95}) of the 42 *Plasmopara viticola* populations to dimethomorph (DMM) and mandipropamid (MPA).

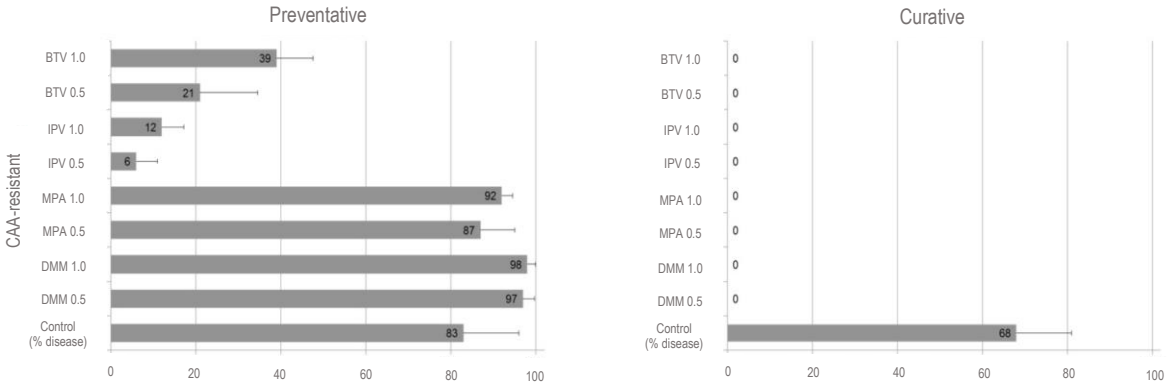

Figure 2 Efficacy of half (0.5) and full (1.0) rate of dimethomorph (DMM), mandipropamid (MPA), iprovalicarb (IPV) and benthiavalicarb (BTV) on a CAA-resistant isolate when applied preventively (left) and curatively (right). Columns and numbers show efficacy for the compounds; for untreated control (control % disease) the value of the diseased (sporulating) leaf area is provided. Error bars represent the standard deviation.

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10 A test method based on the microscopic assessment of CAA fungicides on *P.viticola*

In order to further investigate the findings proposed in chapter 10 (paper II), four carboxylic acid amide fungicides, benthiavalicarb (BTN), dimethomorph (DMM), iprovalicarb (IPR) and mandipropamid (MDP), were examined to test their effectiveness (vitality of sporangia) on *P.viticola* CAA-sensitive and CAA-resistant populations with epifluorescence microscopy utilizing fluorescein diacetate (FDA).

10.1 Material and Methods

10.2 Samples

Vitality assay with FDA were conducted on the CAA-sensitive population (330-BO) and the CAA-resistant population (349-BO) both collected in Italy during the 2015 season and previously screened through detached leaf assay tests. Populations were considered to be sensitive and resistant to mandipropamid and less resistant to dimethomorph based on EC_{50} values (Gisi *et al*; 2007) (Tab 2).

Table 3: EC_{50} values based on sensitivity disc leaf test

Populations	**MDP EC_{50}**	**DMM EC_{50}**
330-BO (S)	0.00	0.44
349-BO (R)	215.82	6.12*

*less resistant

10.3 Sporangial suspension

Sporangial suspensions were obtained by washing off 5 diseased leaves with 5 ml ice cold ddH_2O water with a Pasteur pipette, then the suspension is filtered through cheesecloth. The final concentration utilized for the sensitivity/vitality assay was $1x10^5$ sporangia per ml.

10.4 Fungicides

Benthiavalicarb isopropyl, dimethomorph, iprovalicarb and mandipropamid (Fluka Analytical-Sigma Aldrich,Co –St Lois, Mo-US) were used as technical ingredients. In these assays 0, 1, 5, 25 mg l^{-1} concentrations were tested, as previously prepared as stock solutions in acetone.

10.5 Vitality test

Fungicides were mixed (1:1) with water (as a control) at the sporangial suspension. The microwell containing *P.viticola* sporangia were incubated at 21°-22°C for 1h with slightly agitation. The effect of fungicides on sporangia was determined utilizing 0.4% of fluorescein diacetate using an inverted microscope (Carl Zeiss-Azioskop). Vital sporangia were calculated as a percentage of dead sporangia relative to the total number of sporangia. Fluorescein-diacetate (FDA) stock solution was prepared by dissolving 5 mg FDA per ml acetone and stored at 20 °C until used (Jensen *et al.,* 1995, Miller *et al*. 1998). A phosphate buffer (pH 7.3) was prepared by dissolving 4.6 g dibasic and 2.7 g monobasic potassium phosphate in 100 ml of deionized water. At the time of use, 0.008 ml of the stock FDA solution was added to 2 ml of phosphate buffer. 20 µl of FDA was added in each microwell. The staining solution should always be freshly prepared, not used for longer than 2 hours and kept protected from light. The effect of the four tested CAAs on sporangia was assessed after 1 h. Spores were then examined with an 100x Carl Zeiss compound epiflouresence microscope, equipped with a mercury lamp (HBO50/AC). Positive staining was assessed by the presence of a greenish fluorescence emanating from the cytoplasm of the spores. Quantities of active spores were estimated by counting the number of spores fluorescing out of 100 randomly selected spores for each microwell. Microscopical assays were repeated twice.

10.6 Results and discussion

Table 4 shows the results obtained in terms of percentage of viability utilizing a microscopy method with FDA. The maximum concentration used 25 mg l^{-1} was able to inhibit all sporangia from the sensitive and resistant populations. In the sensitive populations, the concentrations 1 and 5 mg l^{-1} were able to inhibit most of the sporangia, while in the resistant one, the percentage of vitality was still very high. From our early results emerged that the fungicides evaluated showed a cross resistance. FDA can be used for the direct observation in a wide range of spore fungi, therefore this technique was very useful in order to directly observe the activity of these CAAs on *P.viticola* sporangia. This microscopical technique was chosen to collect further results, to complete our findings (*in vitro* and *in vivo* assays), to better consider the CAA sensitivity phenomenon with a different test. In the future, we would like to further investigate by adding other concentrations i.e 7.5, 10, 15, 20 mg l^{-1} to better define the Minimum Inhibitory Concentration (MIC).

Table 4: Percentages of viability of sporangia were calculated as the percentage of dead sporangia relative to the total number of sporangia

Fungicides	**BTN** (mg l^{-1})				**DMM** (mg l^{-1})				**IPV** (mg l^{-1})				**MDP** (mg l^{-1})			
concentration	**0**	**1**	**5**	**25**	**0**	**1**	**5**	**25**	**0**	**1**	**5**	**25**	**0**	**1**	**5**	**25**
S-population (330-BO)	100	45	35	0	100	40	33	0	100	40	30	0	100	39	32	0
R-population (349-BO)	100	82	70	0	100	80	72	0	100	78	75	0	100	80	75	0

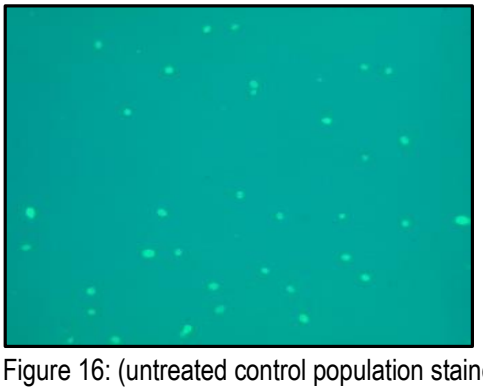

Figure 16: (untreated control population stained with FDA)

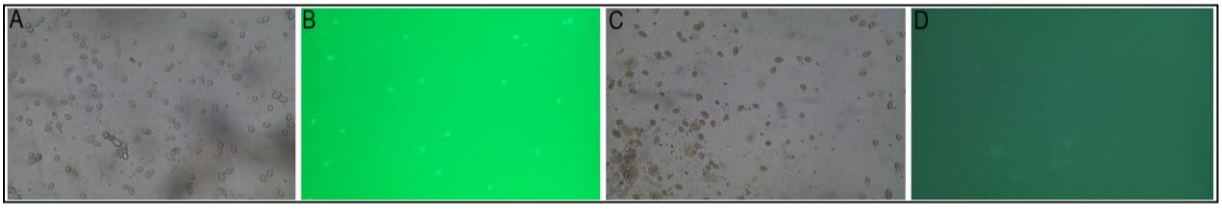

Figure 17: viability test with FDA for the S-population with dimethomorph*; a) 5mg l^{-1} white, b) 5mg l^{-1} with FDA, c) 25mg l^{-1} white, d) 25mg l^{-1} with FDA.

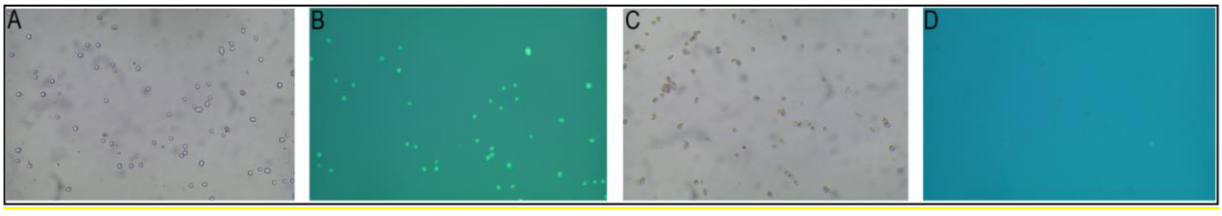

Figure 18: viability test with FDA for the R-population with dimethomorph*; a) 5mg l^{-1} white, b) 5mg l^{-1} with FDA, c) 25mg l^{-1} white, d) 25mg l^{-1} with FDA.

* we report only DMM; as explained in the manuscript (results, section 10.6) all the fungicides showed cross resistance.

11 Mandipropamid and dimethomorph activity on seedling

To confirm our previous findings about the phenomenon of resistance to mandipropamid in Italy, we decided to further study the activity of dimethomorph and mandipropamid on seedlings. With this aim, we randomly selected populations previously tested with disc leaf assay proposed in the paper I, in order to observe how they behaved in a different sensitivity test.

11.1 Material and Methods

11.2 Plant and samples (populations)

Seedlings of *Vitis vinifera* cv Trebbiano were grown in a greenhouse at 22-19° (day-night) with a photoperiod of 15 h of light. The same populations tested with disc leaf assay in the paper in chapter 9 (paper I) were: 276, 287, 291, 292 and G (see Material and Methods chapter 9).

11.3 Greenhouse studies

Three seedlings for each concentration (with 5-7 leaves each) were tested, sprayed with 6 concentrations (1, 3, 10, 30, 100, 300 mg l^{-1} a.i.) of mandipropamid (Pergado SC- Syngenta) and dimethomorph (Forum 50 WP-BASF) till run off**,** 24 hours before inoculation. For each population tested, there was an untreated control included. The inoculation was conducted by spraying a sporangial suspension ($5x10^4$ spores ml^{-1}) onto the adaxial surface of the leaves. The seedlings were then incubated at 100% relative humidity (RH) and 21°C for one day. Diseased leaf area with sporulation was assessed 8-10 days after the inoculation by evaluating the percentage of sporulated leaf area and the EC_{50} values (mg l^{-1}) calculated by probit analysis. Assays were repeated three times.

11.4 Results and discussion

The test performed on the seedlings generally confirmed the results obtained from the disc leaf assay in chapter 9. In fact, from table 5 we can deduce that the activity of mandipropamid toward the populations selected was the same observed in the disc leaf assay (paper I). The dimethomorph EC_{50} values were relatively low when compared to the mandipropamid values. Although these techniques use different approaches and growing conditions are totally different in these two methods, we can confirm that in general, the results obtained were consistent.

Table 5: EC_{50} values from seedlings test and disc leaf assays

Population	Fungicide	EC_{50} (mg l^{-1}) values from seedling test	EC_{50} (mg l^{-1}) values from disc leaf assay (paper I)
276	Mandipropamid	>300	200.28
	Dimethomorph	74.55	6.4
277	Mandipropamid	>300	>300
	Dimethomorph	6.56	0.4
292	Mandipropamid	>300	>300
	Dimethomorph	150	13.22
G	Mandipropamid	0.43	<1
	Dimethomorph	0.45	<1

12 Discussion and conclusions

In paper I, we reported the results of the Italian monitoring developed in order to rapidly screen *Plasmopara viticola* populations coming directly from the field. What we concluded from biological assays were a wide range of EC_{50} values among *P. viticola* Italian populations. Conventional fungicide sensitivity assays for obligate pathogens should be performed *in vivo* i.e. disc leaf assays, whole plants; these test are known to be time consuming, conversely, PCR-RFLP is considered a rapid tool. In this study, PCR-RFLP was able to detect sensitives, resistants and mixed profiles (R/S) among the populations. The PCR-RFLP method was easily reproducible to rapidly screen for the presence of the G1105S mutation involved in the CAA resistance phenomenon. From our data, we could suggest that *P. viticola* populations infecting Italian vineyards are under selection pressure due to carboxylic acid amide based fungicide applications. This relevant information should be taken into account in order to improve current grapevine downy mildew management strategies. In paper II, we illustrated the different levels of efficacy among benthiavalicarb, dimethomorph, iprovalicarb and mandipropamid. Generally, dimethomorph showed lower EC_{95} values as compared to mandipropamid when tested on 42 populations coming from commercial vineyards. All four CAAs were able to fully control the CAA – sensitive strain but the resistant one was controlled at different level by CAAs. Dimethomorph and mandipropamid showed the highest levels of activity, followed by benthiavalicarb and iprovalicarb. The tests also confirmed that all CAAs show curative activity against the CAA-sensitive strain, for within the CAA resistant strain, the curative activity was lost. This difference in preventive and curative control of the CAA-resistant strain could be linked to the mode of action of CAAs. In our preventive trial set up, the tested CAAs controlled the resistant strain differently. Although the cross resistance could be confirmed among these fungicides, we could speculate that the resistance mechanism may differ somewhat between cinnamic acid amides and mandelic acid amide. This hypothesis was also reached by Chen *et a*l. (2012) concerning differences between cinnamic acid amide and valine amide carbamates. We also know from other mode of action groups, such as QoI (Pasche *et al.*, 2005), SDHI (Semar *et al*., 2013) and DMI

(Cools *et al*., 2013), that target site mutations might have a different impact on an individual compounds in the same mode of action group, which may be based on their different chemical structures. This may also be considered for the different CAAs compound. Furthermore, in the viability test performed with FDA, we were able to confirm the cross resistance among all CAAs tested. The sensitive population was controlled by all four fungicides (benthiavalicarb, dimethomorph, iprovalicarb, mandipropamid), starting from the lowest concentrations tested (1 and 5 mg l^{-1}), while the resistant population was controlled by all four CAAs only at highest concentration tested (25 mg l^{-1}). In the seedling test, performed to simulate a scenario closer to the field, we observed that the results were generally consistent with those from bioassays proposed in paper I, although these tests require different approaches. Populations 276 and 277 showed different levels of sensitivity to dimethomorph when tested with different assays. Seedling bioassay is still the most commonly used bioassay for testing sensitivity to fungicides because it closely simulates plants growing in the field. The alternative, quick assays outlined here also detect sensitivity, but the growing conditions are totally different, and for this reason, different EC_{50} values among the same fungicides could be explained. Our bioassays on leaf discs and seedlings also confirmed different behaviors in terms of EC_{50} values between mandipropamid and dimethomorph in the populations tested.

In general, what came out of our study is that among this group of fungicides, in particular among cinnamic acid amide (dimethomorph), mandelic acid amide (mandipropamid) and valinamide carbamates (benthiavalicarb, iprovalicarb), there are some dissimilarities, primarily in chemical structures. Cinnamic acid amides i.e. dimethomorph, flumorph and pyrimorph share the morpholin ring in their structures and this feature could be a starting point for deciphering what we found in our sensitivity studies. Morpholine fungicides belong to a broad group of fungicides, often referred to as sterol inhibitors (SBI). *P.viticola* and other oomycetes belonging to Peronosporales, are unable to synthesize their own sterols and must acquire them from their hosts. As recently as 2010, Gaulin *et al*; deciphered a nearly complete sterol biosynthetic pathway leading to fucusterol in the legume pathogen *Aphanomyces eutechies*, an oomycete belonging to Saprolegniales. Fucosterol was initially characterized in brown

algae and in *A.euteiches* this compound strengthens the phylogenetic relationship between oomycetes and brown algae. The identification of CYP51 enzyme in *A. euteiches* suggests that triazoles could be used against this pathogen. This preliminary experiment paves the way for investigating the potential use of triazoles against *Aphanomyces* diseases, and more generally, against animal and plant diseases caused by oomycetes (Gaulin *et al*; 2010). This finding was particularly significant in highlighting that further investigations about this interesting class of organisms are needed, as they are currently understudied in all areas (Baldauf *et al*., 2000). That said, we cannot exclude the fact that morpholin could act against oomycetes, inhibiting different pathways or helping the main mode of action of this class of fungicides. We also cannot rule out that the morpholin ring in the cinnamic acid amide structure could enforce the activity of this group of fungicides, acting differently from other compounds that lack it. To date, the *P. viticola* genome is not yet sequenced and we are not able to compare it with *Aphanomyces*; the unique Peronosporales genome compared by Gaulin *et al*. (2010) in order to see if Peronosporales were also able to synthesize sterols was *Phytophthora spp*. This annotation of a large Expressed Sequence Tags (EST) collection revelead a series of sequences unique to *A.euteiche*s and absent in *Phytophthora spp.* It is most likely that *P. viticola* will also not reveal the presence of any CYP51 enzyme, because it is placed in the same order of *Phytophthora spp* (Peronosporales), but fucusterol was initially characterized in brown algae, the common ancestor of all oomycetes. Once the *P. viticola* genome is available, it could be very interesting to examine it at a biochemical level to determine if some species of Peronosporales could also have the ability to synthesize sterols. To date we can only speculate if *P. viticola* would have the ability to synthesize sterol, the cinnamic acid amide compound will be able to act not only on one target, such as cellulose biosynthesis, but also on a second one like sterol inhibition. If some mutation occurs causing an alteration of the first biochemical target and resistance to it, the second target might not be involved in the resistance mechanism. In this case, differences between the activity of cinnamic acid amides and mandelic acid amides could be explained. Many kinds of resistance mechanism are known, yet there are still many gaps in our knowledge, not only for

established fungicide groups, but also within new fungicide groups defined by cross-resistance i.e. carboxylic acid amides (Brent *et al.*, 2007). Despite the progress in our work on the first identification of some resistant populations to mandipropamid and the relevant findings on the differences in the activity of CAA in Italy, and although these are grouped in the same class of fungicides, some questions remain to be addressed. Molecular docking studies have been unable to explain the different behavior, because none crystal structure of *P.viticola CesA3* is yet available. Without this information, it's difficult to answer essential questions. This could be done by purifying the *Ces*A3 protein from *P. viticola* and trying to crystallize it. This would be fundamental for predicting the binding mode of all inhibitors in the binding-site and, consequently, deeply examining the changes in the protein due to the mutations occurring in the resistance phenomenon.

13 ACKNOWLEDGEMENTS

My grandpa Ugo passed away when I was abroad for my PhD and it was very difficult to continue my work, but he always said, “you have to find something essential in your research.” For this reason, I would like to dedicate this manuscript to him and to my Grandpa Celso, who passed away many years ago.

I would like to thank my supervisor, Dr. Marina Collina, for giving me the opportunity to develop this project and also to freely improve some ideas to further investigate new findings with different approaches never before applied in this group.

I would also to thank Dr. Gerd Stammler, my co-supervisor, for his collaboration during these years, and for reading the manuscript and giving me important advice.

I would like to thank Maria, Sasha, Marco, and Violeta, my EMBL friends, who were very close to me during my Hamburg period and are still now, despite the distance….

I would like to acknowledge Daniela and Marco P., who helped a lot with the fungicides sensitivity assays, and my lab colleagues, for supporting me.

I also thank Luca my PhD course mate to make the coffee break always very funny!

A special thank goes to my boyfriend and colleague Alessandro, who helped me with experiments, giving me significant suggestions and supporting me in every moment.

Last but not least I would like to thank all of my family, who are very important for me and always close to my heart. And thanks to my pets, Manny and Schell ….

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